

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

As rescanning documents *will not* correct images,  
please do not report the images to the  
**Image Problem Mailbox.**



---

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/82, C07K 14/415</b>		(11) International Publication Number: <b>WO 97/35023</b>
(21) International Application Number: <b>PCT/US97/04409</b>		(43) International Publication Date: <b>25 September 1997 (25.09.97)</b>
(22) International Filing Date: <b>19 March 1997 (19.03.97)</b>		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BI, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(30) Priority Data: <b>08/618,911</b> <b>20 March 1996 (20.03.96)</b> <b>US</b>		
(71) Applicant: <b>PIONEER HI-BRED INTERNATIONAL, INC.</b> <b>[US/US]; 7100 N.W. 62nd Avenue, P.O. Box 1000, Johnston, IA 50131-1000 (US).</b>		
(72) Inventors: <b>JUNG, Rudolf; 1549 Germania Drive, Des Moines, IA 50311 (US). HASTINGS, Craig; 2315 Iowa Street, Perry, IA 50220 (US). COUGHLAN, Sean; 5610 Waterbury Circle, Des Moines, IA 50312 (US). HU, David; 5611 Kensington Circle, Johnston, IA 50131 (US).</b>		
(74) Agents: <b>JERVIS, Herbert et al.; 7100 N.W. 62nd Avenue, P.O. Box 1000, Johnston, IA 50131-1000 (US).</b>		<b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: ALTERATION OF AMINO ACID COMPOSITIONS IN SEEDS

**ALI** N-terminal amino acid sequence by Edman degradation

**BEWEGUNGSGESTALTUNG**  
small chain

**YINXINGCHENGJIEGONGQITONG**  
Joint chain

#### AI2 N-terminal amino acid sequence by Edman degradation

**SKWPGQPSQMSKSDLSKCNLPTFZM**  
small chain

YUWAKINGCHENGCHENQK7752495LK  
large china

**7500** **(pA1.42, partial aa sequence deduced from cDNA)**

[illegible]

**ALS** (in sequence by Edman degradation)

SKWOBQDSZKOLQGVNLSPTNCHDME  
small chole

NCXCDHNEHCGLDAGQK77  
large chain

**p7881** (**pA1.1\_02**, partial as sequence deduced from cDNA)

**REKRD00SCIKOLOGVNLTCRHMENKQCRGDDDDDDNNILKTRGERTYLRNCKDIDRNCHDKOKCTMRRL**

**(57) Abstract**

The present invention provides methods for increasing the level of preselected amino acids in seeds of plants, thereby enhancing the nutritional value of the seeds, by genetic modification. The present invention is particularly useful in increasing the methionine, lysine, and/or cysteine content in seeds of plants. Also provided, are isolated endogenous DNA molecules which encode soybean albumins. The present invention also provides an antibody which is capable of specifically binding to soybean albumins. The present invention further provides methods for isolating and purifying 2S albumins.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroun	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## ALTERATION OF AMINO ACID COMPOSITIONS IN SEEDS

BACKGROUND OF THE INVENTION

5

Feed formulations based on crop plants must typically be supplemented with specific amino acids to provide animals with essential nutrients which are critical to their growth. This supplementation is necessary because, in general, crop  
10 plants contain low proportions of several amino acids which are essential for, and cannot be synthesized by, monogastric animals.

The seeds of crop plants contain different classes of seed proteins. The amino acid composition of these seeds  
15 reflects the composition of the prevalent classes of proteins. Amino acid limitations are usually due to amino acid deficiencies of these prevalent protein classes.

Among the amino acids necessary for animal nutrition, those that are of limited availability in crop plants  
20 include methionine, lysine and cysteine. For example, in soybean, the 7S globulin accounts for about 30% of the seed proteins but contains only 0.3% of methionine, whereas the Bowman-Birk inhibitor ("BBI") accounts for approximately 1% of seed proteins but contains approximately 20% sulfur  
25 containing amino acids. Attempts to increase the levels of these amino acids by breeding, mutant selection, and/or changing the composition of the storage proteins accumulated in the seeds of crop plants, have met with limited success, or were accompanied by a loss in yield.

30 For example, although seeds of corn plants containing a mutant transcription factor, (opaque 2), or a mutant  $\alpha$ -zein gene, (floury 2), exhibit elevated levels of total and bound lysine, there is an altered seed endosperm structure which is more susceptible to damage and pests. Significant yield  
35 losses are also typical.

An alternative means to enhance levels of free amino acids in a crop plant is the modification of amino acid biosynthesis in the plant. The introduction of a feedback-regulation-insensitive dihydrodipicolinic acid synthase ("DHDPS") gene, which encodes an enzyme that catalyzes the first reaction unique to the lysine biosynthetic pathway, into plants has resulted in an increase in the levels of free lysine in the leaves and seeds of those plants. However, these increases are insufficient to significantly increase the total amino acid content of the seed because the level of free amino acid in seeds is, in general, only a minor fraction of the total amino acid content.

The expression of the *lysC* gene, which encodes a mutant bacterial aspartate kinase that is desensitized to feedback inhibition by lysine and threonine, from a seed-specific promoter in tobacco plants, has resulted in an increase in methionine and threonine biosynthesis in the seeds of those plants. See Karchi, et al.; The Plant J.; Vol. 3; p. 721; (1993); incorporated herein in its entirety by reference. However, expression of the *lysC* gene results in only a 6-7% increase in the level of total threonine or methionine in the seed. Thus, the expression of the *lysC* gene in seeds has a minimal impact on the nutritional value of those seeds and, thus, supplementation of feed containing *lysC* transgenic seeds with amino acids, such as methionine and threonine, is still required.

There are additional molecular genetic strategies available for enhancing the amino acid quality of plant proteins. Each involves molecular manipulation of plant genes and the generation of transgenic plants.

Protein sequence modification involves the identification of a gene encoding a major protein, preferably a storage protein, as the target for modification to contain more codons of essential amino acids. A critical task of this approach is to be able to select a region of

the protein that can be modified without affecting the overall structure, stability, function, and other cellular and nutritional properties of the protein. The variable region(s) in a polypeptide, as identified through sequence analysis and comparison of related protein species, offer possible target sites for such modifications.

These studies indicate both that it is feasible to increase the essential amino acid residues in a seed protein by sequence modifications, and that it is important to select suitable target sites.

The development of DNA synthesis technology allows the design and synthesis of a gene encoding a new protein with desirable essential amino acid compositions. For example, researchers have synthesized a 292-base pair DNA sequence encoding a polypeptide composed of 80% essential amino acids and used it with the nopaline synthetase (NOS) promoter to construct a chimeric gene. Expression of this gene in the tuber of transgenic potato has resulted in an accumulation of this protein at a level of 0.02% to 0.35% of the total plant protein. This low level accumulation is possibly due to the weak NOS promoter and/or the instability of the new protein.

A protein of minor quantity in a plant may contain elevated levels of an essential amino acid that is limiting. By enhancing the expression of the gene encoding this protein, it may be possible to increase the concentration of this protein, and thus the content of this particular essential amino acid. In this connection, a 10.8-kD putative methionine-rich protein has recently been considered in soybean seeds as a good candidate for improving the protein quality of soybeans.

Additionally, recombinant DNA and plant transformation techniques permit the transfer of genes between diverse plant species. Thus, a gene encoding an essential amino acid-rich protein isolated from a specific plant can be

introduced into other plants to enhance their protein quality. Several plant proteins containing unusually high levels of the essential sulfur amino acids and their genes have been identified and isolated. They are prime  
5 candidates for use in protein improvement.

Tobacco has been used as a test plant to demonstrate the feasibility of this approach by transferring a chimeric gene containing the bean phaseolin promoter and the cDNA of a sulfur-rich protein Brazil Nut Protein ("BNP"), (18 mol%  
10 methionine and 8 mol% cysteine) into tobacco. Amino acid analysis indicates that the methionine content in the transgenic seeds is enhanced by 30% over that of the untransformed seeds. This same chimeric gene has also been transferred into a commercial crop, canola, and similar  
15 levels of enhancement were achieved.

However, an adverse effect is that lysine content decreases. Additionally, BNP has been identified as a major food allergen. Thus it is neither practical nor desirable to use BNP to enhance the nutritional value of crop plants.

20 This finding marks an area that needs further research. It is also useful to point out here that there are advantages and disadvantages to each of these approaches. While the protein sequence modification and the synthetic gene strategies have the flexibility of engineering and  
25 designing a gene with desirable essential amino acid composition, they suffer from the possibility of generating unknown structural and biological properties in the protein product. Both the heterologous and homologous gene approaches enjoy the advantage of utilizing naturally-  
30 occurring genes. However, the identification of a gene encoding a protein rich in a particular essential amino acid, if it indeed exists, could be a formidable task.

There is therefore a need to change the ratio of protein classes, without detrimental side effects.  
35 Endogenous proteins are well adapted for intracellular

assembly, targeting and processing. Additionally, a change of the protein composition reduces the possibility of generating unknown risks for human or animal health because all protein compounds are already present in the plant prior to modification. However, some endogenous proteins, such as BBI, which are rich in essential amino acids, are anti-nutritional proteins.

Based on the foregoing, there exists a need to identify endogenous seed storage proteins with increased amounts of essential amino acids, which are present in relatively low amounts in unmodified seeds, to enhance the nutritional value of seeds by genetically modifying the seeds so as to over-express genes encoding these proteins. The genetic modification should not be accompanied by detrimental side effects such as allergenicity, anti-nutritional quality or poor yield.

It is therefore an object of the present invention to provide methods for increasing the nutritional content of feed.

It is a further object of the present invention to provide methods for genetically modifying seeds so as to increase amounts of essential amino acids present in relatively low amounts in unmodified seeds.

It is a further object of the present invention to provide methods for introducing endogenous proteins into seeds.

It is a further object of the present invention to provide methods for increasing the nutritional content of seeds without detrimental side effects such as allergenicity, poor yield or anti-nutritional quality.

#### SUMMARY OF THE INVENTION

The methods of the present invention comprise the transformation of plant cells by introducing an expression



cassette comprising a preselected DNA segment encoding a seed storage protein.

The present invention also provides a fertile transgenic soybean plant containing an isolated preselected DNA segment comprising a promoter and encoding a seed storage protein comprising preselected amino acids under the control of the promoter.

The present invention also provides an isolated and purified DNA molecule comprising a preselected DNA segment encoding a soybean seed storage protein.

The present invention also provides an antibody capable of specifically binding soybean albumin.

The present invention also provides methods of isolating albumins from seeds.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the amino-terminal sequences of albumin 1, albumin 2, and albumin 3, as determined by Edman degradation of proteins isolated from PVDF blots.

Figure 2 depicts the cDNA sequence (SEQ ID NO: 1) of albumin 1 isolated from a soybean seed cDNA library, and the corresponding predicted amino acid sequence of albumin 1 (SEQ ID NO: 2).

Figure 3 depicts the cDNA sequence (SEQ ID NO: 3) of albumin 3 isolated from a soybean seed cDNA library, and the corresponding predicted amino acid sequence of albumin 3 (SEQ ID NO: 4).

Figure 4 depicts the cDNA sequence (SEQ ID NO: 5) and the amino acid sequence (SEQ ID NO: 6) of a chimeric albumin which comprises sequences from albumin 1 and albumin 3

Figure 5 termed albumin 1/3 depicts a comparison of the amino acid sequences of albumin 1, albumin 3 and albumin 1/3.

Figure 6 depicts a plasmid map of p4752.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for genetically modifying seeds to increase the level of at least one preselected amino acid in the seed so as to enhance the nutritional value of the seeds. The methods comprise the introduction of an expression cassette into regenerable plant cells to yield transformed plant cells. The expression cassette comprises a preselected DNA segment, encoding a soybean seed storage protein comprising preselected amino acids, operably linked to a promoter functional in plant cells.

A fertile transgenic plant is regenerated from the transformed cells, and seeds are isolated from the plant. The seeds comprise the protein which is encoded by the preselected DNA segment and which is produced in an amount sufficient to increase the amount of the preselected amino acid in the seeds of the transformed plants, relative to the amount of the preselected amino acid in the seeds of a corresponding untransformed plant, e.g., the seeds of a regenerated control plant that is not transformed or corresponding untransformed seeds isolated from the transformed plant.

Preferably, the preselected amino acid is lysine. More preferably, there is an additional preselected amino acid. Even more preferably, the additional preselected amino acid is cysteine or methionine.

A preferred embodiment of the present invention is the introduction of an expression cassette into regenerable soybean cells. Also preferred is the introduction of an expression cassette comprising a preselected DNA segment encoding an endogenous polypeptide sequence.

The present invention encompasses segments having sufficient similarity to the segments disclosed hereinafter. Generally, such sufficient similarity should comprise at least about 60% identity or 60% homology between base pairs

10 through 474 in albumin 1 (SEQ ID NO: 1), between base pairs 28 through 501 in albumin 3 (SEQ ID NO: 3) and between base pairs 28 and 501 in albumin 1/3 (SEQ ID NO: 5). Preferably, such sufficient similarity should comprise at least about 70% identity or 70% homology. More preferably, such sufficient similarity should comprise at least about 80% identity or 80% homology. Even more preferably, such sufficient similarity should comprise at least about 90% identity or 90% homology. Most preferably, the segments of the present invention are of the sequences disclosed in SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5 respectively.

The present invention also encompasses variations in the sequences described above, wherein such variations are due to site-directed mutagenesis, or other mechanisms known in the art, to increase or decrease levels of selected amino acids of interest. For example, site-directed mutagenesis to increase levels of lysine, methionine and/or cysteine, and/or to decrease levels of asparagine and/or glutamine is a preferred embodiment.

The present invention also provides a fertile transgenic plant. The fertile transgenic plant contains an isolated preselected DNA segment comprising a promoter and encoding a seed storage protein comprising preselected amino acids under the control of the promoter. The DNA segment is expressed as the seed storage protein so that the level of preselected seed storage protein amino acids in the seeds of the transgenic plant is increased above the level in the seeds of a plant which only differ from the seeds of the transgenic plant in that the DNA segment or the encoded seed protein is under the control of a different promoter. The DNA segment is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

Also provided is an isolated and purified DNA molecule comprising a preselected DNA segment encoding a soybean seed storage protein. A most preferred embodiment of the

invention is a preselected DNA segment encoding a soybean albumin. See e.g. Shewry, et al.; The Plant Cell; Vol. 7; No. 7; pp. 945-956; (1995); incorporated herein in its entirety by reference.

5       The present invention also provides an expression cassette comprising a preselected DNA segment encoding a soybean seed storage protein, operably linked to a promoter functional in a host cell. Preferred promoters useful in the practice of the invention are those seed-specific  
10 promoters that allow expression of the preselected DNA segment selectively in seeds to avoid any potential deleterious effects associated with the expression of the preselected DNA segment in non-seed organs.

Other embodiments of the invention include plants,  
15 plant parts, seeds and microorganisms transformed with the preselected DNA segment encoding a seed storage protein. Preferably, the seed storage protein is an albumin. More preferably, the seed storage protein is a soybean albumin.

Other embodiments of the present invention also include  
20 a chimera with increased levels of preselected amino acids.

In a preferred embodiment of the present invention, a method is provided for the simple, rapid, and reliable production of transgenic soybean plants with increased accumulation of lysine, in the seeds produced thereby. In a  
25 more preferred embodiment, increased accumulation of methionine and/or cysteine occurs in addition to increased accumulation of lysine. The method is genotype-independent and shows a substantial, unexpected improvement over previously used systems.

30       The present invention also provides methods for isolating and purifying 2S albumins comprising the separation of albumins from contaminants by specifically interacting the albumins with the matrix of a carbohydrate resin, preferably a dextran resin, even more preferably  
35 Sephadex G25. The above methods for isolation and

purification are unexpected given the molecular sieve characteristics of the resin. The specific interaction between the albumins and the matrix has applications useful for batch processes.

5 As used herein, a "preselected DNA segment" means an exogenous or recombinant DNA sequence or segment that encodes a soybean seed storage protein, wherein the seed storage protein is preferably not a functional protease inhibitor, not a functional  $\alpha$  amylase inhibitor and not a  
10 lectin.

A preferred seed storage protein of the invention is one that has an increased content of lysine as well as sulfur containing amino acids, i.e., methionine and/or cysteine. The choice of the preselected DNA segment and  
15 amino acid is based on the amino acid composition of the protein encoded by the preselected DNA segment, and the ability of the protein to accumulate in seeds. Moreover, the amino acid composition of the protein can be manipulated by methods, such as site-directed mutagenesis of the  
20 preselected DNA segment encoding the protein, so as to result in expression of a protein that is increased in the amount, i.e., content, of a particular amino acid. A preferred embodiment of the invention is a preselected DNA segment encoding a soybean seed storage protein that has an  
25 elevated amount of lysine, and methionine and/or cysteine, such as a preselected DNA segment encoding a soybean albumin. Because an endogenous protein is utilized, the possibility of generating unknown risks for human and/or animal health is reduced.

30 As used herein, the term "high lysine content protein" means that the protein has at least about 7% lysine, more preferably at least about 10% lysine, even more preferably at least about 12% lysine, and most preferably at least about 13% lysine. In a preferred embodiment, the high

lysine content protein is also a high sulfur content protein.

As used herein, the term "high sulfur content protein" means that the protein contains methionine and/or cysteine in addition to lysine, at levels indicated hereinafter. The high sulfur content protein has at least about 6% methionine and/or cysteine, preferably at least about 9% methionine and/or cysteine, and more preferably at least about 11% methionine and/or cysteine.

As used herein, "increased" or "elevated" levels or amounts of preselected amino acids in a transformed plant are levels which are greater than the levels or amounts in the corresponding untransformed plant. For example, the average methionine content in soybean seed proteins is about 1.4%, the average cysteine content in soybean seed proteins is about 1.4%, and the average lysine content in soybean seed proteins is about 6.0% (George, et al.; J. Agric. Food Chem.; Vol. 34; p. 224; (1991); incorporated herein in its entirety by reference). Thus, the expression of soybean albumin 1 having SEQ ID NO: 2, which has about 12% of a combination of methionine and cysteine and about 10% lysine, in seeds results in an increase in the level or amount of methionine, cysteine and lysine in those seeds. Furthermore, the expression of soybean albumin 3, having SEQ ID NO: 4, which has about 12% of a combination of methionine and cysteine and about 10% lysine, in seeds results in an increase in the level or amount of methionine, cysteine and lysine in those seeds. The amino acid composition of a protein can be determined by methods well known to the art.

Increased amounts of preselected amino acids other than lysine in a transformed plant are preferably at least about 15 to 30%, preferably at least about 30 to 50%, and most preferably about 50 to 100%, greater than the amounts of the preselected amino acid in a non-transformed plant. Increased amounts of preselected lysine in a transformed

plant are preferably at least about 5-10%, more preferably at least about 10-15%, even more preferably at least about 15-25%, most preferably at least about 25-50% greater than the amounts of lysine in a non-transformed plant.

5 As used herein, "genetically modified plant" means a plant which comprises a preselected DNA segment which is introduced into the genome of the plant by transformation. The term "wild type" refers to an untransformed plant i.e., one where the genome has not been altered by the  
10 introduction of the preselected DNA segment.

As used herein, "plant" includes but is not limited to plant cells, plant tissue and plant seeds. For the present invention, preferred plants include soybean, canola, sunflower, sorghum and corn. More preferred plants include  
15 soybean and corn. The most preferred plant is soybean.

As used herein with respect to a preselected DNA segment encoding a protein, the term "expresses" means that the preselected DNA segment is incorporated into the genome of the cells, so that the product encoded by the preselected  
20 DNA segment, e.g., a sulfur-rich protein such as albumin, is produced within the cells. For example, novel plants resulting from expression of a preselected DNA segment encoding an albumin contain extractable levels of the albumin of at least about 3%, preferably at least about 5%,  
25 more preferably at least about 10%, and even more preferably at least about 20%, of the total protein in the seed.

The class of plants which can be used in the method of the invention is generally as broad as the class of seed-bearing higher plants amenable to transformation techniques,  
30 including both monocotyledonous and dicotyledonous plants. Seeds derived from plants regenerated from transformed plant cells, plant parts or plant tissues, or progeny derived from the regenerated transformed plants, may be used directly as feed or food, or further processing may occur. In the  
35 practice of the present invention, the most preferred plant

seed is selected from that of soybean, canola, sunflower, sorghum and corn. More preferably, the plant seed is that of corn or soybean, most preferably that of the soybean *Glycine max*. The transformation of the plants in accordance  
5 with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. These include, but are not limited to, microprojectile bombardment, microinjection, electroporation of protoplasts or cells comprising partial cell walls, and  
10 *Agrobacterium*-mediated DNA transfer.

As used herein, "recombinant" DNA is a DNA sequence or segment that has been isolated from a cell, purified, or amplified.

As used herein, "isolated" means either physically  
15 isolated from the cell or synthesized *in vitro* on the basis of the sequence of an isolated DNA segment.

As used herein, "albumin" means a seed protein whose genes encode peptide precursors similar in organization to and homologous to the 2S albumin seed protein family. See  
20 Shewry supra ; incorporated herein in its entirety by reference.

As used herein, "2S soybean albumin" means a Glycine seed protein whose genes encode peptide precursors which are homologs of the albumins.

25 The present invention provides for the expression of a protein of preselected amino acid composition in a seed at levels sufficient to reduce or obviate feed supplementation. A preferred protein, which is encoded by a preselected DNA segment of the invention, is a seed storage protein.  
30 Because seed storage proteins normally accumulate in seed, overexpression of these proteins in seed will not have to overcome incompatibility with the assembly, targeting and processing mechanisms in the cell. In addition, there is minimal risk of enhancement of induction of allergenic  
35 reactions in comparison with wild type seeds. A preferred



embodiment of the invention includes a seed storage protein rich in lysine as well as sulfur-containing amino acids. One example of such a protein is an albumin. To enhance expression of a protein of preselected amino acid composition in a seed at a level to increase the level of the preselected amino acid in the seed, expression cassettes with seed-specific promoters can be employed.

#### I. DNA USED FOR TRANSFORMATION

DNA-encoding seed storage protein(s) useful for introduction into plant cells includes DNA that has been derived or isolated from any source, that may be subsequently characterized as to structure, size and/or function, chemically altered, and later introduced into the plant. An example of DNA "derived" from a source, would be a DNA sequence or segment that is identified as a useful fragment within a given organism, and which is then synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from the source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

Therefore, useful DNA includes completely synthetic DNA, semi-synthetic DNA, DNA isolated from biological sources, and DNA derived from RNA. The DNA isolated from biological sources, or DNA derived from RNA, includes, but is not limited to, DNA or RNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses. The DNA or RNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different genotype. The term "chimeric gene" or "chimeric DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not recombine DNA under natural conditions, or which DNA sequences or segments are

positioned or linked in a manner which does not normally occur in the native genome of untransformed plant. Thus, it is within the scope of the invention to isolate a preselected DNA segment from a given soybean genotype, and  
5 to subsequently introduce at least one copy of the preselected DNA segment into the same genotype.

A preselected DNA segment of the invention can be identified by standard methods, e.g., enrichment protocols, or probes, directed to the isolation of particular  
10 nucleotide or amino acid sequences. The preselected DNA segment can be identified by obtaining and/or screening of a DNA or cDNA library generated from nucleic acid derived from a particular cell type, cell line, primary cells, or tissue. Screening for DNA fragments that encode all or a portion of  
15 the preselected DNA segment can be accomplished by screening plaques from a genomic or cDNA library for hybridization to a probe of the preselected DNA segment from other organisms or by screening plaques from a cDNA expression library for binding to antibodies that specifically recognize the  
20 protein encoded by the preselected DNA segment. DNA fragments that hybridize to a preselected DNA segment probe from other organisms and/or plaques carrying DNA fragments that are immunoreactive with antibodies to the protein encoded by the preselected DNA segment can be subcloned into  
25 a vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion of the preselected DNA segment.

Portions of the genomic copy or copies of the preselected DNA segment can be partially sequenced and  
30 identified by standard methods including either DNA sequence homology to other homologous genes or by comparison of encoded amino acid sequences to known protein sequences. Once portions of the preselected DNA segment are identified, complete copies of the preselected DNA segment can be  
35 obtained by standard methods, including cloning or

polymerase chain reaction (PCR) synthesis using oligonucleotide primers complementary to the preselected DNA segment. The presence of an isolated full-length copy of the preselected DNA segment can be verified by comparison of its deduced amino acid sequence with the amino acid sequence of native polypeptide sequences.

The preselected DNA segment encoding the seed storage protein can be modified to increase the content of particular amino acid residues in that protein by methods well known to the art, including, but not limited to, site-directed mutagenesis. Thus, derivatives of naturally occurring proteins can be made by nucleotide substitution of the preselected DNA segment encoding that protein so as to result in a protein having a different amino acid at the position in the protein which corresponds to the codon with the nucleotide substitution. The introduction of multiple amino acid changes in a protein can result in a protein which is significantly enriched in a preselected amino acid.

The present invention thus provides a DNA molecule comprising a preselected DNA segment encoding a seed storage protein. The preselected DNA segment can encode any seed storage protein including, but not limited to, the 2S, 7S and 11S seed storage proteins, with or without modification of the sequence encoding those proteins. The skilled artisan will recognize that the choice of the protein encoded by the preselected DNA segment will be based on the amino acid composition of the protein and its ability to accumulate in seeds. The amino acid can be chosen for its nutritional value to produce a value-added trait to the plant or plant part. Amino acids desirable for value-added traits, as well as a source to limit synthesis of an endogenous protein include, but are not limited to, methionine, cysteine, and lysine.

Also provided are methods for increasing the level of at least one preselected amino acid in seeds by expressing a

preselected DNA segment encoding a protein in seeds. Preferably, the preselected amino acid is lysine. More preferably, a second preselected amino acid is also included in the present invention. Even more preferably, the second  
5 preselected amino acid is methionine or cysteine. Expression of the preselected DNA segment, or multiple copies of the preselected DNA segment, can increase the level of the protein encoded by the preselected DNA segment in the seeds and, thus, the level of the preselected amino  
10 acid which has been incorporated into the protein encoded by the preselected DNA segment. Methods and compositions are provided for producing plant cultures, plant tissues, plants and seeds that comprise an expression cassette comprising a preselected DNA segment encoding a protein. The present  
15 invention provides a method of genetically engineering plants so that the plants produce seeds with increased levels of at least one preselected amino acid, such that plants and seeds can sexually transmit this trait to their progeny.

20 In a preferred embodiment, the protein encoded by the preselected DNA segment is a sulfur rich 2S seed storage protein, such as albumin. In a more preferred embodiment of the invention, the preselected DNA segment encodes an endogenous 2S soybean albumin. By way of example, and not  
25 limitation, those skilled in the art will readily appreciate that the 2S albumin gene from other organisms may be substituted for the soybean 2S albumin protein. See, for example, Coulter, et al.; J. Exp. Bot.; Vol. 41; p. 1541; (1990); incorporated herein in its entirety by reference.

30 Other examples of sulfur-rich plant proteins within the scope of the invention include plant proteins enriched in cysteine but not methionine, such as the wheat endosperm purothionine (Mak and Jones; Can. J. Biochem.; Vol. 22; p. 83J; (1976); incorporated herein in its entirety by  
35 reference), and the pea low molecular weight albumins

(Higgins, et al.; J. Biol. Chem.; Vol. 261; p. 11124; (1986); incorporated herein in its entirety by reference). Such proteins also include methionine-rich plant proteins such as from sunflower seed (Lilley, et al.; In: Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs; Applewhite, H. (ed.); American Oil Chemists Soc.; Champaign, IL; pp. 497-502; (1989); incorporated herein in its entirety by reference), corn (Pedersen, et al.; J. Biol. Chem. p. 261; p. 6279; (1986); Kirihara, et al.; Gene, Vol. 71; p. 359; (1988); both incorporated herein in its entirety by reference), and rice (Musumura, et al.; Plant Mol. Biol.; Vol. 12; p. 123; (1989); incorporated herein in its entirety by reference).

#### Expression Cassettes and Expression Vectors

According to the present invention, a preselected DNA segment encoding a protein, such as a seed storage protein, is identified, isolated, and combined with at least a promoter functional in a host cell, e.g., a plant cell, to provide a recombinant expression cassette. The construction of such expression cassettes which may be employed in conjunction with the present invention are well known to those of skill in the art in light of the present disclosure. See, e.g., Sambrook, et al.; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor, New York; (1989); Gelvin, et al.; Plant Molecular Biology Manual; (1990); Plant Biotechnology: Commercial Prospects and Problems, eds Prakash, et al.; Oxford & IBH Publishing Co.; New Delhi, India; (1993); and Heslot, et al.; Molecular Biology and Genetic Engineering of Yeasts; CRC Press, Inc., USA; (1992); each incorporated herein in its entirety by reference.

#### Promoters

Preferred expression cassettes of the invention will generally include, but are not limited to, a seed-specific promoter. Examples of seed-specific promoters include promoters of seed storage proteins which express these

proteins in seeds in a highly regulated manner (Thompson, et al.; BioEssays; Vol. 10; p. 108; (1989); incorporated herein in its entirety by reference), such as, for dicotyledonous plants, a bean  $\beta$ -phaseolin promoter, a napin promoter, a  $\beta$ -conglycinin promoter, and a soybean lectin promoter. For monocotyledonous plants, promoters useful in the practice of the invention include, but are not limited to, a maize 15 kD zein promoter, a 22 kD zein promoter, a  $\gamma$ -zein promoter, a waxy promoter, a shrunken 1 promoter, a globulin 1 promoter, and the shrunken 2 promoter. However, other promoters useful in the practice of the invention are known to those of skill in the art.

## II. DELIVERY OF DNA TO CELLS

The expression cassette or vector can be introduced into prokaryotic or eukaryotic cells by currently available methods. For example, the expression cassette or vector can be introduced into plant cells by methods including, but not limited to, *Agrobacterium*-mediated transformation, electroporation, microprojectile bombardment, microinjection, infectious viruses or viroids, the use of liposomes and the like, all in accordance with well-known procedures. Plant cells useful for transformation include cells cultured in suspension cultures, callus, embryos, meristem tissue, pollen, and the like. Transformed cells can be selected typically using a selectable or screenable marker encoded on the expression vector.

Introduction and expression of foreign genes in dicotyledonous plants such as soybean, tobacco, potato and alfalfa has been shown to be possible using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. Using recombinant DNA techniques and bacterial genetics, a wide variety of foreign DNAs can be inserted into T-DNA in *Agrobacterium*. Following infection by the bacterium containing the recombinant Ti plasmid, the foreign DNA is

inserted into the host of plant chromosomes, thus producing a genetically engineered cell and eventually a genetically engineered plant. A second approach is to introduce root-inducing (Ri) plasmids as the gene vectors.

5 While *Agrobacterium* appear to preferably attack dicots, many important crop plants including maize, wheat, rice, barley, oats, sorghum, millet, and rye are monocots and are not known to be easily susceptible to transformation by *Agrobacterium*. The Ti plasmid, however, may be manipulated  
10 in the future to act as a vector for monocot plants. Additionally, using the Ti plasmid as a model system, it may be possible to artificially construct transformation vectors for monocot plants. Ti-plasmids might also be introduced into monocots by artificial methods such as microinjection,  
15 or fusion between monocot protoplasts and bacterial spheroplasts containing the T-region, which can then be integrated into the plant nuclear DNA. Other transformation methods are readily available to those skilled in the art.

### III. REGENERATION AND ANALYSIS OF TRANSFORMANTS

20 Following transformation, regeneration is involved to obtain a whole plant from transformed cells and the presence of preselected DNA segment(s) or "transgene(s)" in the regenerating plant detected by assays. The seed derived from the plant is then tested for levels of preselected  
25 amino acids. Depending on the type of plant and the level of gene expression, introduction of the preselected DNA segment into the plant can enhance the level of preselected amino acids in an amount useful to supplement the nutritional quality of those seeds.

30 Techniques for regenerating plants from tissue culture, such as transformed protoplasts or callus cell lines, are known in the art. For example, see Phillips, et al.; Plant Cell Tissue Organ Culture; Vol. 1; p. 123; (1981); Patterson, et al.; Plant Sci.; Vol. 42; p. 125; (1985);  
35 Wright, et al.; Plant Cell Reports; Vol. 6; p. 83; (1987);

and Barwale, et al.; Planta; Vol. 167; p. 473; (1986); each incorporated herein in its entirety by reference. The selection of an appropriate method is within the skill of the art.

5 Examples of the practice of present invention detailed herein relate specifically to soybean plants and expression  
vectors operable in dicots. However, the present invention  
is also applicable to other plants. The expression vectors  
utilized herein are demonstrably capable of operation in  
10 cells of many dicotyledonous plants both in tissue culture  
and in whole plants. The invention disclosed herein is thus  
operable in dicotyledonous species to transform individual  
plant cells and to achieve full, intact plants in dicot  
plant species which can be regenerated from transformed  
15 plant cells and which express preselected seed storage  
proteins.

The introduced preselected DNA segments are expressed  
in the transformed plant cells and stably transmitted  
(somatically and sexually) to the next generation of cells  
20 produced. The vector should be capable of introducing,  
maintaining, and expressing a preselected DNA segment in  
plant cells. Additionally, it is possible to introduce the  
vector into a wide variety of cells of plants. The  
preselected DNA segment is passed on to progeny by normal  
25 sexual transmission.

To confirm the presence of the preselected DNA  
segment(s) or "transgene(s)" in the regenerating plants, or  
seeds or progeny derived from the regenerated plant, a  
variety of assays may be performed. Such assays include,  
30 for example, "molecular biological" assays well known to  
those of skill in the art, such as Southern and Northern  
blotting and PCR; "biochemical" assays, such as detecting  
the presence of a protein product, e.g., by immunological  
means (ELISAs and Western blots) or by enzymatic function;  
35 plant part assays, such as leaf, seed or root assays; and



also, by analyzing the phenotype of the whole regenerated plant.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA may only be  
5 expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques may also be used for detection and quantitation of RNA produced from introduced preselected DNA segments. In this application of PCR it is first necessary  
10 to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the  
15 nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern  
20 hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the preselected DNA segment in question, they do not provide  
25 information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced preselected DNA segments or evaluating the phenotypic changes brought about by their expression.

30 Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic  
35 procedures, such as native or denaturing gel electrophoresis

or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Very frequently, the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms, including but not limited to, analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of preselected DNA segments encoding storage proteins which change amino acid composition and may be detected by amino acid analysis.

Breeding techniques useful in the present invention are well known in the art.

The present invention has been described with reference to various specific and preferred embodiments and will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention.

EXAMPLE 1Isolation and Characterization of Soybean 2S Albumins

Soybean plants (G. max Merr.) varieties are grown in the greenhouse or in the field. If not otherwise stated, 5 reagents and laboratory supplies are obtained from Sigma Chemical Co. (St. Louis, MO) or Baxter (McGaw Park, IL). Protein concentrations are estimated either according to Bradford (BioRad® protein assay, BioRad®, Hercules, CA) or with a modified Lowry assay (DC protein assay, BioRad®) 10 with bovine serum albumin (Pierce, Rockford, IL) as a standard.

The present method comprises the steps of:

- a) protein extraction from soybean meal;
- b) size exclusion chromatography of the protein 15 extract;
- c) collection of albumin-containing fractions;
- d) separation of albumins from other protein contaminants by specifically interacting the albumins to the matrix of a resin; and
- 20 e) ion-exchange chromatography to separate the individual albumins.

SDS polyacrylamide gel electrophoresis ("PAGE") is performed using the Tris-Tricine buffer system developed by Schagger and von Jagow. See Schagger, H. and von Jagow, G., 25 Anal. Biochem. Vol. 166, p. 368 (1987); incorporated herein in its entirety by reference. For routine purposes polypeptides are separated in 16.5% Mini-Protein II precast mini-gels (80x73 mm Ready Gels, BioRad®, Richmond, CA) or, when a superior resolution of polypeptides in the molecular 30 weight range between 2 and 25 kDa is required, in 170x150 mm 8-22% polyacrylamide gradient gel using a Model V16 electrophoresis apparatus (GibcoBRL®, Gaithersburg, MD). Protein bands are detected by staining with Coomassie brilliant Blue R 250.

When indicated, after electrophoretically separating the proteins, the separated polypeptides are electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon PSQ®, Millipore, Bedford, MA) using a semi-dry electroblotter (SemiPhor® TE70, Hoefer, San Francisco, CA) as described by Matsudaira (J. Biol. Chem.; Vol. 262; p. 10035; (1987); incorporated herein in its entirety by reference). Several precautions are undertaken to prevent blocking of N-terminal amino groups and to minimize modifications of amino acid side chains prior to amino acid sequencing. The Tris/Tricine gels, including the stacking gel, are cast 3-7 days prior to the protein separation and stored sealed at 4°C. Immediately before separation, gels are pre-run at 2V/cm for 15 hours with 0.1% SDS, 0.75M Tris/HCl, pH 8.45 (anode buffer) and 0.1% SDS, 1M Tris/HCl, pH 8.45 (cathode buffer). Following electrotransfer of polypeptides to PVDF (see above) and staining with Coomassie Blue, the blots are washed extensively with water and dried. Polypeptide bands of interest are carefully excised from the membranes and stored in microcentrifuge tubes at 4°C until needed. N-terminal sequence is obtained from Immobilon PSQ membranes by using an Applied Biosystems 477A Protein sequencer in the Protein Analysis Laboratory of the University of Iowa (Iowa City, IA).

Amino acid analysis is carried out on a Beckman 6300 analyzer according to standard procedures. Methionine and cysteine are determined as methionine sulfone and cysteic acid after performic acid oxidation. Isoelectric focusing of proteins is performed in pre-cast slab gels (pH performance range 3.5-6.5, Novex, San Diego, CA) with Novex Low Range IEF protein standards according to the manufacturer's recommendations.

To determine whether an isolated protein contains N-linked glycans, Concanavalin A-horseradish peroxidase staining of protein blots (see above) is carried out as described by Faye and Chrispeels (Anal. Biochem.; Vol. 149, p. 218; (1985); incorporated herein in its entirety by reference) with the modification that horseradish peroxidase activity is visualized by chemiluminescence (ECL kit, Amersham, Arlington Heights, IL).

For N-Glycosidase F (Boehringer Mannheim, Indianapolis, IN) treatment (0.1 U/10  $\mu$ l, 15 hours at 37°C) protein samples (10  $\mu$ g/10  $\mu$ l) are denatured by 2 min. at 95°C in 0.1% SDS, 200 mM NaCl, 20 mM Tris/HCl, pH 8.5, cooled to 4°C, supplemented to 1% Triton X-100 and incubated for 15 min. at room temperature prior to enzyme addition.

**Purification and Characterization of Lysine-rich and Sulfur-rich Soybean 2S Albumins**

Transgenic soybean seed expressing a methionine-rich 2S seed storage protein from Brazil Nut (Bertholletia excelsa) ("BNP") shows a reduction in the levels of the sulfur-rich endogenous Bowman-Birk inhibitor (Kollipara, K.P. and Hymowitz, R.; J. Agri. Food; Vol. 40; pp. 2356-2363; (1992); incorporated herein in its entirety by reference) and the reduction of an unknown 14 kDa protein. To determine whether the unknown 14 kDa protein is a methionine-rich seed storage protein, seed proteins from wild type seeds and BNP transgenic seeds are electrophoretically separated and electrotransferred to PVDF membranes, and then the membranes are probed with iodo[<sup>14</sup>C]acetic acid (ICN Radiochemicals, Irvine, CA), pH 2.0, according to the method of de Lumen and Kho (J. Agric. Food Chem.; Vol. 35; p. 688; (1987); incorporated herein in its entirety by reference). An autoradiogram of this gel blot shows that the 14 kDa protein is a methionine-rich protein. This protein may belong to a family of methionine-containing peptides previously observed

by Kho and de Lumen (Plant Food Hum. Nutr.; Vol. 38; p. 287; (1988); incorporated herein in its entirety by reference) using the same technique.

To purify this protein, mature dry seed of soybean  
5 (Glycine max) is ground into a fine meal, defatted by extraction with hexane (1:1 w/v) and vacuum dried. 100 g of defatted flour is homogenized in a Waring blender for 5 min. at 4° C with 400 ml 10% DMSO, 0.5% n-butanol, 100 mM KCL, 83 mM sodium acetate buffer, pH 5.2, (albumin extraction  
10 buffer). All following steps are carried out either on ice or at 4° C.

The slurry is filtered through Miracloth® (Calbiochem, LaJolla, CA) and centrifuged at 6000 Xg for 15 min. The recovered supernatant is dialyzed (Spectra/por 7, MWCO 3500,  
15 Baxter, McGaw Park, IL) extensively against 0.5% n-butanol, 100 mM KCL, 83 mM sodium acetate buffer, pH 5.2 and concentrated in the dialysis bags to about 100 ml with dry polyethyleneglycol (PEG 8000). Precipitated contaminating globulin proteins are removed by centrifugation at 6000 Xg  
20 for 15 min. and by filtration through a 0.45 µm membrane. The resulting albumin extract contains approximately 20% of the total seed protein. 5-10% of the albumin fraction is represented by the 14 kDa polypeptides which comprises approx. 1-2% of the total soybean seed protein (0.5-1% of  
25 the seed weight) in wild-type seeds. The extractability in dilute acidic buffer classifies the 14 kDa proteins as albumins (Osborne, The Vegetable Proteins, Longman, G. (ed.), London (1924); incorporated herein in its entirety by reference). The 14 kDa protein dissociates in SDS PAGE  
30 under reducing conditions into two polypeptides, apparently of 10 kDa and of 5 kDa respectively, indicating linkage by disulfide bridges in the holoprotein.

Five ml of the concentrated albumin extract (conc. approx. 20 mg/ml), is further fractionated using an Superdex

75 HiLoad 26/60 column (Pharmacia, Uppsala, Sweden) which is run with the extraction buffer. The flow rate is maintained at 1 ml/min. and fractions of 4 ml are collected and analyzed by PAGE. The fractions containing the putative  
5 albumin (fraction 33-35, 18 mg protein), obtained with approximately 50% purity and Kunitz trypsin inhibitor (KTI) (Kollipara supra) as the major contaminant, are adjusted to pH 8.5 with Tris HCl (1 M) and chromatographed using a 100 ml Sephadex G 25 sf column (Pharmacia, Uppsala, Sweden) with  
10 50 mM sodium acetate, pH 5.2, running buffer at 1 ml/min.

The 14 kDa proteins exhibit, under these conditions, an unexpected interaction with the dextran matrix of the column and separate from its protein contaminants as a single peak with more than 95% purity. A similar specific interaction  
15 with the dextran matrix can be observed with the 2S albumin from Brazil Nut and can be used for its purification in a single step. Other albumins also behave in a similar manner. Other carbohydrate matrices known to the skilled artisan may similarly be used in the process. Though the  
20 above mentioned chromatography step has been specifically described, it can be replaced by other techniques involving specific interactions, e.g., but not limited to batch processes.

The above-obtained putative albumin fraction is dialyzed  
25 (Spectra/por 7) for 15 hours against 20 mM Tris/HCl pH 8.5 and concentrated in the dialysis bags to about 0.5 mg/ml protein with dry PEG 8000. 5 mg of the desalted protein is filtered through a 0.2  $\mu$ m membrane filter and fractionated further by ion-exchange chromatography using a MonoQ HR 5/5  
30 (Pharmacia, Uppsala, Sweden) column, developed in a gradient of 0-750 mM NaCl in 20 mM Tris/HCl, pH 8.5 buffer. Three separate peaks, elute at 180 mM NaCl (designated albumin 1), 250 mM NaCl (designated albumin 2), and 360 mM NaCl (designated albumin 3). Albumin 3 (Al3) appears to be the  
35 major form, i.e. it contains more than 90% of the protein in

all three fractions combined, whereas albumin 1 (Al1) and albumin 2 (Al2) are found to be each approximately 20 times less abundant among the soybean seed proteins when compared to Al 3. All three albumin fractions are obtained at near  
5 homogeneity based on SDS-PAGE. After treatment with the reducing agent 2-mercapto-ethanol, each of the three albumin forms dissociated in SDS PAGE into two smaller polypeptides of different length, indicating the presence of disulfide bonds in the native protein. The sizes of the larger  
10 peptides in each of the reduced albumins appear to be similar (10 kDa), whereas the shorter peptides appear to be of different sizes. The Al1 small chain has an estimated molecular weight of 4.5 kDa, the Al2 small chain of 4.8 kDa and the Al3 small chain of 5.1 kDa, respectively.  
15 PVDF blots of the electrophoretically separated Al2 large chain, Al2 small chain, Al3 large chain and Al3 short chain are subjected to amino acid analysis (Table 1). Both albumins contain the predicted high contents of methionine and in addition, a surprisingly high percentage of lysine.  
20 Although it appears that the amino acid compositions of the two albumins are generally similar, some clear differences with some amino acids are observed.



**TABLE 1**  
**AMINO ACID COMPOSITION**

		A12		A13	
		5 kDa Peptide Mole %	11 kDa Peptide Mole %	5 kDa Peptide Mole %	11 kDa Peptide Mole %
5	Cys	1.93	3.38	2.79	2.88
	Asx	10.89	8.17	17.96	9.47
10	Met	3.13	8.00	2.35	8.70
	Thr	1.76	1.03	4.10	3.02
	Ser	9.62	9.00	7.05	7.43
	Glx	21.86	19.39	15.42	21.80
	Pro	0.00	2.65	3.67	3.02
15	Gly	14.01	9.43	5.85	6.64
	Ala	12.99	10.72	5.29	11.10
	Val	0.00	0.00	3.63	0.42
	Ile	6.59	5.90	4.46	4.07
	Leu	5.33	8.96	6.84	8.32
20	Tyr	0.38	0.64	2.45	0.00
	Phe	0.76	0.54	1.90	0.31
	His	2.93	1.11	3.09	1.24
	Lys	4.24	8.11	6.43	8.85
	Arg	3.58	2.96	6.80	1.85

The amino terminal sequence of all small and large chain peptides is determined from PVDF blots of the respective electrophoretic peptide bands by automated Edman-degradation in an Applied Biosystems sequencer. The amino-terminal sequences of albumin 1 and 2 are identical. The amino-terminal sequences of albumin 3 are different than those of albumin 1 and 2. However, the amino-terminal sequences of albumin 3 have a high degree of homology to the amino-terminal sequences of albumin 1 and 2 (about 80%). These amino-terminal sequences are most closely related to sequences found in conglutin  $\delta$ , a sulfur rich 2S protein from *Lupinus angustifolius* L (Gayler, et al.; Plant Mol. Biol.; Vol. 15; p. 879; (1990); incorporated herein in its entirety by reference).

To elucidate the differences between the A1 peptides, the albumin fractions are further analyzed by isoelectric focusing. The isoelectric point of A11 is determined at a pH of 6.05, of A12 at a pH of 5.45 and of A13 at a pH of 4.95, respectively.

Since the cDNA specific for A11 and A13 encode a consensus sequence for asparagine linked N-glycosylation, (see below), concanavalin A binding to albumin-containing fractions is analyzed. None of the A11 peptides bind concanavalin A, nor are molecular weight size differences apparent in SDS PAGE after N-Glycosidase F treatment. Therefore, N-glycosylation of soybean albumins seems unlikely.

The protein sequencing data, together with the amino acid composition results, indicates the occurrence of the following distinct yet undescribed methionine- and lysine-rich albumin gene products, i.e. A11 and A12, and A13, in soybean seeds. The similarity of the N-terminal A11 and A12 amino acid sequences can be explained by the assumption of differential post-translational processing events of the same gene product.

## EXAMPLE II.

Isolation of Albumin-specific cDNA Clones  
from a Soybean Seed cDNA Library RNA isolation,  
cDNA synthesis and sequence analysis.

5 DNA isolation, DNA manipulations, radiolabelling of DNA and hybridizations are done essentially as described by Sambrook, et al.; Molecular Cloning: A Laboratory Manual, Cold Spring Harbor (1989); incorporated herein in its  
10 entirety by reference.

Soybean plants (*Glycine max* Merr.) are grown in the greenhouse or in the field. Developing, mid-maturation soybean seeds are harvested and stored frozen at -80°C to be used as a mRNA source for cDNA library construction.

15 Total RNA is isolated from pooled developing soybean seed (1-15 mm in size). Frozen seeds (1-2 g fresh weight) are ground to a powder in a pestle and mortar, and RNA is isolated according to methods described in Shure, et al.; Cell; Vol. 35; p. 225-233; (1983); incorporated herein in  
20 its entirety by reference. mRNA is isolated from 1 mg total RNA using an oligo-dT Sepharose spin column according to the manufacture's instructions (Pharmacia, Uppsala, Sweden). Five µg of purified mRNA is used as a template for cDNA  
25 synthesis and ligation into Stratagene Lambda Zap II vector arms according to the manufacturer's instructions (Stratagene, La Jolla, CA). One hundred ng of size selected cDNA (>500 bp) is ligated to the vector arms and packaged (Stratagene Gigapack Gold) to yield a primary library of  $1.2 \times 10^6$  pfu with an average cDNA insert size of 1.2 kb. This  
30 library is amplified in *E. coli* Sure cells (Stratagene) to give a titre of  $2 \times 10^{10}$  pfu/ml.

Two hundred random plaques are isolated and re-suspended in 500 µl SM. Phagemids (Bluescript S/K) are excised from the Lambda ZAP II vector according to the  
35 protocol recommended by Stratagene using helper phage R408

and *E. coli* host strain XL1 Blue. Single colonies are grown overnight in 2 ml of 2 x YT medium containing 100 µg/ml ampicillin. The plasmid DNA is isolated by alkaline lysis and ethanol precipitation (Sambrook, et al.; Supra; (1989);  
5 incorporated herein in its entirety by reference).

The 5' sequence from 200 individual cDNA clones is obtained using the T3 primer by Taq cycle sequencing on an ABI catalyst 8000 Molecular workstation and ABI 1373A sequenator (Applied Biosystems). Sequence data is edited  
10 manually to remove vector sequence and a database of the DNA sequence information from the 200 randomly picked cDNA clones using this library is created to facilitate the identification and isolation of cDNA clones encoding abundant expressed polypeptide sequences for which the  
15 albumin polypeptides would be an example.

#### Identification of Albumin Specific cDNA Clones

The cDNA database is searched with back-translated DNA sequences corresponding to the amino terminal sequences of the A11/2 and A13 small and large chains using the FASTA®  
20 algorithm (Genetics Computer Group, Wisconsin sequence analysis package, Version 8). A section of the deduced amino acid sequence of clone EST 3\_38 is found to display an exact match to the amino terminal sequences derived from the small and large chains of A11. The deduced amino acid  
25 sequences of clone EST 2\_36, clone EST 3\_13, clone EST 3\_14 and clone EST 3\_62 are found to align exactly with the obtained amino terminal sequences of the A13 peptides. Furthermore, a computer comparison of the deduced amino acid sequence of clone EST 3\_38 and of clone 3\_62 with the  
30 Genbank sequence data base using the TFasta® algorithm (Genetics Computer Group) reveal homology to conglutin δ, a sulfur-rich 2S protein from lupin seeds (*Lupinus angustifolius* L.). (Gayler, et al.; Supra; (1990); incorporated herein in its entirety by reference).

An about 600 bp EcoRI fragment from clone EST 3\_38 and an about 400 bp EcoRI/SacI fragment from clone EST 3\_62 are labeled with [<sup>32</sup>P] dCTP (Amersham) using the Ready Prime kit from Amersham. The labeled fragments are used to screen  
5 15,000 recombinant phages from the cDNA library derived from developing soybean seeds in Lambda ZapII (Stratagene). Approximately 3% of the clones in the library hybridize to both albumin probes.

Forty-five albumin specific phages are randomly  
10 selected and the corresponding phagemids are subsequently excised according to the manufacturer's recommendations and sequenced. Among the sequenced clones, 42 are found to be albumin 3 specific (7 encoding the entire coding sequence) and 3 are found to be albumin 1 specific (one encoding the  
15 entire coding sequence).

The inserts of the longest identified Al1 and Al3 specific clones, pAl1\_42 and pAl3\_49, respectively, are sequenced in their entirety (Figures 2 and 3) and consequently entered into the Pioneer plasmid collection  
20 under the names p9330 and p9331 respectively. Sequence analysis clearly identifies that these clones contain full-length coding sequences, encoding both the N-terminal signal peptides and the stop codon.

Albumin 1 is encoded by 465 base pairs comprised in a  
25 723 base pair cDNA (SEQ ID NO: 1). This cDNA encodes a pre-propeptide having 155 amino acids (SEQ ID NO: 2). The pre-propeptide comprises a 20 amino acid signal peptide, about a 55 amino acid small chain, and about a 80 amino acid large chain. The mature albumin protein comprises two disulfide  
30 linked chain, a 4-5 kDa small chain and a 10 kDa large chain. The amino acid composition of the deduced amino acid. Sequence of albumin 1 includes 11.8 mol% methionine and cysteine residues, 9.6 mol% lysine residues and 12.6 mol% asparagine and glutamine residues.

Albumin 3 is encoded by 474 base pairs comprised in a 777 base pair cDNA (SEQ ID NO: 3). This cDNA encodes a pre-propeptide having 158 amino acids (SEQ ID NO: 4). The pre-propeptide comprises a 21 amino acid signal peptide, about a 60 amino acid small chain and a 77 amino acid large chain. The mature albumin 3 contains two disulfide linked chains. The deduced amino acid composition of albumin 3 includes 11.6 mol% methionine and cysteine residues, 10.2 mol% lysine residues, and 13.2 mol% asparagine and glutamine residues.

#### EXAMPLE III.

To further enhance for the preferred amino acid residues lysine and methionine and to further reduce non-preferred amino acid residues asparagine and glutamine, a cDNA encoding a chimeric albumin, termed albumin 1/3 (Al 1/3) (SEQ ID NO: 6), is prepared based on a GAP alignment (Genetics Computer Group) of the amino-acid sequences of Al1 and Al3 (Fig. 5).

The cDNA clone p9331 (pAl3\_49) is modified by oligodeoxyribonucleotide-directed mutagenesis using the Muta-Gene Phagemid in vitro Mutagenesis kit from BioRad (Hercules, CA) based on the Kunkel method (Kunkel, T.A., Proc. Nat. Acad. Sci. USA, Vol. 82; p. 488; (1985); incorporated herein in its entirety by reference) according to the manufacturer's recommendations. Mutagenesis is carried out in five consecutive repetitions of in vitro mutagenesis with five oligodeoxyribonucleotide primers. The primers and the changes they confer to the cDNA sequence are summarized in Table 2.

TABLE 2

## MUTAGENIC OLIGODEOXYRIBONUCLEOTIDE PRIMERS

SEQ. ID NO:	Oligodeoxyribonucleotide Sequence	Position of Mutagenized Amino Relation to the Encoded A13 Prepropeptide	Amino Acid Codon Changed
7	5'GCTGCCGCAAGCAGCTTAAGGGGGTGAACCTC3'	36	Gln to Lys
8	5'GGAAGAATCAACTACATACGTAAAGGAAGGAAAGACG3'	80	Arg to Lys
9	5'GCTGCACAGAAATGAGCGAGCTTAAGAGCCCCCAAATGCCAGTGC3'	81	Asn to Lys
10	5'GGAGGAGAGGAGAGAAAGAAATGGAGAAGGAGTTCATGAACCTTGGC3'	105	Arg to Lys
		129	Gln to Glu
11	5'GCAGGTTTGGGCCCCATGATCGGGTGGACTTGTCCTC3'	138	Ile to Met
		151	Gln to Gly

The amino acid codons at the indicated positions of the cDNA encoding Al3 are essentially only changed into codons which encode preferred amino acids found at the same relative positions (GAP alignment) in the protein sequence of Al1. Thus the resulting amino acid sequence Al 1/3 is termed a chimeric albumin. All changes of amino acid residues are made in sequence regions which are considered important for the protein structure of related 2S albumins from seeds of other plant species and are therefore not obviously amenable for a change. Nevertheless, because the amino acid residues in Al 1/3 are already present in either Al1 or Al3, the structure of the chimeric protein is unlikely to exhibit any deleterious effects when expressed in a seed. Albumin 1/3 has 158 amino acids (Fig. 6). The amino acid composition of albumin 1/3 includes 12.4 mol% methionine and cysteine residues, 13.14 mol% lysine residues, and 10.3 mol% asparagine and glutamine residues.

#### EXAMPLE IV.

#### Transformation of *Glycine max* with High Lysine Content and High Sulfur Content Storage Protein Genes

Soybean (*Glycine max*) seed, is surface sterilized by exposure to chlorine gas evolved in a glass bell jar. Gas is produced by adding 3.5 ml hydrochloric acid (34-37% w/w) to 100 ml sodium hypochlorite (5.25% w/w). Exposure is for 16-20 hours in a container approximately one cubic foot in volume. Surface sterilized seed is stored in petri dishes at room temperature. Seed is germinated by plating on 1/10 strength agar solidified medium according to Gamborg, et al.; (Exp. Cell. Res.; Vol. 50, pp. 151-158; (1968); incorporated herein in its entirety by reference). (B5 basal medium with minimal organics, Sigma Chemical Co., Cat. no. G5893; 0.32 g/L; sucrose, 0.2% w/v and 2-[N-morpholino]ethanesulfonic acid (MES), 3.0mM) without plant growth regulators and culturing at 28°C with a 16 hour day length and cool white fluorescent illumination of



approximately 20 mEm<sup>2</sup>S<sup>1</sup>. After three or four days, seed is prepared for co-cultivation. The seed coat is removed and the elongating radical is removed 3-4mm below the cotyledons. Ten prepared seeds are held in each of several petri dishes.

#### Construction of Plant Gene Expression Cassettes

The expression cassette containing one copy of a soybean albumin gene under the control of phaseolin regulatory sequences is the binary plasmid p9127. p9127 is constructed in several steps beginning with oligodeoxynucleotide directed mutagenesis of p9330 (pAl1\_42) which contains the full-length coding sequence of the All protein in the plasmid backbone of Bluescript SK (Stratagene®). Mutagenesis is carried out as described in Example III with oligodeoxyribonucleotide:

- 1) 5'GCACGAGTCATGACCAAGTCACAATTCTC 3' (SEQ ID NO: 12); and
- 2) 5'TCCTCCGATGACTGAGTTAACAAAAAAGTACTAC 3' (SEQ ID NO: 13);

so that an RcaI site is placed and a HindIII site is destroyed at the start of translation and an HpaI site is added just 3' of the stop codon. Upon digestion with the restriction endonucleases RcaI/HpaI, a 472 base-pair DNA sequence corresponding to the full length coding sequence of All is isolated and cloned into p4752 (NcoI/HpaI). p4752 (Fig. 6) contains 883 base pairs of the phaseolin 5' regulatory sequences (i.e. promoter) followed by 84 base pairs of the phaseolin 5' untranslated region. Immediately 3' to these sequences are an NcoI site and HpaI site to facilitate cloning in the 5' → 3' direction of an open reading frame resulting in the codon methionine start translation generated by the NcoI site (- CCATGG) becoming the translational start codon. Downstream of the HpaI site is 1230 base pairs of phaseolin 3' regulatory sequences. p4752 thus contains the phaseolin promoter: phaseolin terminator.

The resulting plasmid, p9069 is then digested with the restriction endonucleases EcoRI/HindIII and the phaseolin promoter: All:phaseolin terminator portion is inserted into the EcoRI/HindIII site of plasmid p1830 (=pARC12) (Prosen, et al.; Biotechnology; Vol. 5; p. 966; (1987); incorporated herein in its entirety by reference). Plasmid p1830 is a 29.5 kb plasmid which is part of a binary vector system of *Agrobacterium* and contains the chimeric gene nopaline synthase/neomycine phosphotransferase II as a selectable marker for plant cells.

The plasmid resulting after the insertion of the 2.89 kb fragment of p9069 inserted into p1830 is termed p9127. Plasmid p9127 is about 33 kb in size and confers resistance to tetracycline to the bacterial host.

The plasmid is then transformed to *Agrobacterium tumefaciens* strain LBA 4404 by the freeze/thaw method, known in the art. The presence of the binary plasmid in the resulting bacteria is confirmed by Southern blot analysis.

#### Preparation of *Agrobacterium tumefaciens* LBA 4404/p9127

Overnight culture of *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary plasmid p9127 grown to log phase in Minimal A medium containing tetracycline, 1.0 mg/ml, is pooled and an optical density measurement at 550 nm is taken. Sufficient volume of the culture is placed in 15 ml conical centrifuge tubes such that upon sedimentation between  $1.0$  and  $2.0 \times 10^{10}$  cells are collected in each tube, where O.D.550 of  $1.0 = 1.4 \times 10^9$  cells/ml. Sedimentation is by centrifugation at 6000 g for 10 minutes. After centrifugation the supernatant is decanted and the tubes are held at room temperature until inoculum is needed, but not longer than one hour.

#### Transformation

Inoculations are conducted in batches such that each plate of seed is treated with a newly resuspended pellet of

*Agrobacterium*. One at a time, the pellets are resuspended in 20 ml inoculation medium. Inoculation medium consist of B5 salts (Sigma Chemical Co.), 3.2 g/L; sucrose, 2.0% w/v 6-benzylaminopurine (BAP), 44 mM; indolebutyric acid (IBA), 0.5 mM; acetosyringone (AS), 100 mM and is buffered to pH 5.5 with MES, 10 mM. Resuspension is by vortexing. The inoculum is then poured into a petri dish containing prepared seed and the cotyledonary nodes are macerated with a surgical blade. This is accomplished by dividing seed in half by longitudinal section through the shoot apex preserving the two whole cotyledons. The two halves of the shoot apex are then broken off their respective cotyledons by prying them away with a surgical blade. The cotyledonary node is then macerated with the surgical blade by repeated scoring along the axis of symmetry. Care is taken not to cut entirely through the explant to the adaxial side. Twenty explants are prepared in roughly 5 minutes and then incubated for 30 minutes at room temperature without agitation. Additional plates are prepared during this time. After 30 minutes the explants are transferred to plates of the same medium solidified with Gelrite (Merck & Co., Inc.), 0.2% w/v. Explants are embedded with the adaxial side up and level with the surface of the medium and cultured at 22°C for three days under cool white fluorescent light, approximately 20 mEm<sup>2</sup>S<sup>1</sup>.

#### Culture and Selection

After three days the explants are moved to liquid counterselection medium. Counterselection medium consists of B5 sales, 3.2 g/L; sucrose, 2.0% w/v; BAP, 5.0 mM; IBA 0.5 mM; vancomycin, 200 mg/ml; cefotaxime, 500 mg/ml and is buffered to pH 5.7 with MES, 3 mM. Ten explants are washed in each petri dish with constant, slow gyratory agitation at room temperature for four days. Counterselection medium is replaced four times.

The explants are then picked to agarose solidified selection medium. Selection medium consists of B5 salts, 3.2 g/L; sucrose, 2.0%, w/v; BAP, 5.0 mM; IBA, 0.5 mM; kanamycin sulfate, 50 mg/ml and is buffered to pH 5.7 with MES, 3.0 mM. Selection medium is solidified with SeaKem agarose, 0.3% w/v. The explants are embedded in the medium, adaxial side down and cultured at 28°C with a 16 hour day length and cool white fluorescent illumination of 60-80 mEm<sup>2</sup>S<sup>-1</sup>.

After two weeks explants are again washed with liquid medium on the gyratory shaker. This time the wash is conducted overnight in counterselection medium containing kanamycin sulfate, 50 mg/ml. The following day explants are picked to agarose solidified selection medium. Again they are embedded in the medium, adaxial side down; the culture is as before for another two week period.

#### Regeneration

After one month on selective media transformed tissue becomes visible as green sectors of regenerating tissue against a background of bleached, less healthy tissue. Explants without green sectors are discarded, explants with green sectors are transferred to elongation medium. Elongation medium consists of B5 salts, 3.2 g/L; sucrose, 2.0% w/v; IBA, 3.3 mM; gibberellic acid, 1.7 mM; vancomycin, 100 mg/ml; cefotaxime, 30 mg/ml; and timentin, 30 mg/ml, buffered to pH 5.7 with MES, 3.0 mM. Elongation medium is solidified with gelrite, 0.2% w/v. They are embedded adaxial side up and cultured as before. Culture is continued on this medium with transfer to fresh plates every two weeks. When shoots become 0.5 cm in length they are excised at the base and placed in rooting medium in 13 x 100 mm test tubes. Rooting medium consists of B5 salts, 3.2 g/L; sucrose, 15 gm/L; nicotinic acid, 20 mM; pyroglutamic acid (PGA), 900 mg/L and IBA, 10 mM. It is buffered to pH 5.7 with MES, 3.0mM and solidified with Gelrite, 0.2% w/v.

After ten days the shoots are transferred to the same medium without IBA or PGA. Shoots are rooted and held in these tubes under the same environmental conditions as before.

When a root system is well established, the plantlet is transferred to sterile soil mix in plant cons (ICN Biomedicals, Inc., Irvin, CA, cat no. 26-720 & 1-02). Temperature, photoperiod and light intensity remain the same as before. Under these conditions the regenerates become vigorous, mostly normal (though small) plants. When their root systems again become well established, a corner of the plant cone is cut off and the plants are gradually hardened off in an environmental chamber or greenhouse. Finally they are potted in soil mix and grown to maturity, bearing seed, in a greenhouse.

#### 15 Growth, Increase, and Harvest of Transgenic Systems

Seed from untransformed and transformed plants of the same variety is planted in the spring and harvested in the fall. Each individual line is kept separate while grown in one or more 10.5 foot rows for maximum increase.

20 The determination of the levels of a particular protein can be determined by methods well known in the art including, but not limited to enzyme linked immunoassays, immunofluorescent assays, Western blot analysis and immunoprecipitation analyses.

25 The amino acid content of seeds from transformed and untransformed plants is analyzed by methods described in the Office Methods of Analysis of the AOAC, Hilrich (ed.), AOAC International; Vol. 2; p. 1096; (1990); incorporated in its entirety by reference.

#### 30 EXAMPLE IV

##### Preparation of Albumin-specific Antibodies

Antibodies specific for albumin polypeptides are produced by injecting female New Zealand white rabbits (Bethyl Laboratory, Montgomery, TX) six times with homogenized polyacrylamide gel slices containing 100 µg of

PAGE purified albumin. Animals are then bled at two week intervals. The antibodies are further purified by affinity-chromatography with Affigel 15(BioRad)-immobilized antigen as described by Harlow, et al.; Antibodies: A Laboratory Manual, Cold Spring Harbor, NY; (1988); incorporated herein  
5 in its entirety by reference. The affinity column is prepared with purified albumin 3 essentially is recommended by BioRad®. Immune detection of antigens on PVDF blots is carried out following the protocol of Meyer, et al.; J. Cell. Biol.; Vol. 107; p. 163; (1988); incorporated herein  
10 in its entirety by reference, using the ECL kit from Amersham (Arlington Heights, IL).

All publications and patents are incorporated by reference herein, as though individually incorporated by  
15 reference. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) ADDRESSEE: PIONEER HI-BRED INTERNATIONAL, INC.  
 (B) STREET: Darwin Bldg., 7100 N.W. 62nd Ave.  
 (C) CITY: Johnston  
 (D) STATE: Iowa  
 (E) COUNTRY: USA  
 (F) ZIP: 50131-1000

(ii) TITLE OF INVENTION: ALTERATION OF AMINO ACID COMPOSITIONS IN SEEDS

(iii) NUMBER OF SEQUENCES: 13

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT: Unassigned  
 (B) FILING DATE: Concurrently herewith

## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/613,911  
 (B) FILING DATE: 20-MAR-1996

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 723 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 10..474

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCACGAGAA ATG ACC AAG CTT ACA ATT CTC CTC ATC GCT CTT CTC TTC	48
Met Thr Lys Leu Thr Ile Leu Leu Ile Ala Leu Leu Phe	
1 5 10	
ATC GCC CAC ACC TGC TGC GCC TCC AAA TGG CAA CAG CAC CAG CAA GAG	96
Ile Ala His Thr Cys Cys Ala Ser Lys Trp Gln Gln His Gln Gln Glu	
15 20 25	
AGC TGC CGC GAG CAG CTC AAG GGG ATC AAC CTC AAC CCC TGT GAG CAC	144
Ser Cys Arg Glu Gln Leu Lys Gly Ile Asn Leu Asn Pro Cys Glu His	
30 35 40 45	
ATC ATG GAG AAG ATC CAA GCT GGC CGC CGC GGC GAG GAC GGC AGC GAC	192
Ile Met Glu Lys Ile Gln Ala Gly Arg Arg Gly Glu Asp Gly Ser Asp	
50 55 60	

(2) INFORMATION FOR SEQ ID NO:2:

(A) LENGTH: 155 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

45



Glu Met Ser Glu Leu Lys S r Pro Ile Cys Gln Cys Lys Ala Leu Gln  
 100 105 110  
 Lys Ile Met Asp Asn Gln Ser Glu Gln Leu Glu Gly Lys Glu Lys Lys  
 115 120 125  
 Gln Met Glu Arg Glu Leu M t Asn Leu Ala Ile Arg Cys Arg Leu Gly  
 130 135 140  
 Pro Met Ile Gly Cys Asp Leu Ser Ser Asp Asp  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 777 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 28..501

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGCTCGTGC CGAATCGGCA CGAGAAA ATG ACC AAG TTC ACA ATC CTC CTC 51  
 Met Thr Lys Phe Thr Ile Leu Leu  
 160  
 ATC TCT CTT CTC TTC TGC ATC GCC CAC ACT TGC AGC GCC TCC AAA TGG 99  
 Ile Ser Leu Leu Phe Cys Ile Ala His Thr Cys Ser Ala Ser Lys Trp  
 165 170 175  
 CAG CAC CAG CAA GAT AGC TGC CGC AAG CAG CTC CAG GGG GTG AAC CTC 147  
 Gln His Gln Gln Asp Ser Cys Arg Lys Gln Leu Gln Gly Val Asn Leu  
 180 185 190 195  
 ACG CCC TGC GAG AAG CAC ATC ATG GAG AAG ATC CAA GGC CGC GGC GAT 195  
 Thr Pro Cys Glu Lys His Ile Met Glu Lys Ile Gln Gly Arg Gly Asp  
 200 205 210  
 GAC GAT GAT GAT GAT GAC GAC GAC AAT CAC ATT CTC AGG ACC ATG CGG 243  
 Asp Asp Asp Asp Asp Asp Asp Asp Asn His Ile Leu Arg Thr Met Arg  
 215 220 225  
 GGA AGA ATC AAC TAC ATA AGG AGG AAC GAA GGA AAA GAC GAA GAC GAA 291  
 Gly Arg Ile Asn Tyr Ile Arg Arg Asn Glu Gly Lys Asp Glu Asp Glu  
 230 235 240  
 GAA GAA GAA GGA CAC ATG CAG AAG TGC TGC ACA GAA ATG AGC GAG CTG 339  
 Glu Glu Glu Gly His Met Gln Lys Cys Cys Thr Glu Met Ser Glu Leu  
 245 250 255  
 AGA AGC CCC AAA TGC CAG TGC AAA GCG CTG CAG AAG ATA ATG GAG AAC 387  
 Arg Ser Pro Lys Cys Gln Cys Lys Ala Leu Gln Lys Ile Met Glu Asn  
 260 265 270 275  
 CAG AGC GAG GAA CTG GAG GAG AAG CAG AAG AAG AAA ATG GAG AAG GAG 435  
 Gln Ser Glu Glu Leu Glu Glu Lys Gln Lys Lys Lys Met Glu Lys Glu  
 280 285 290 295

CTC ATT AAC TTG GCT ACT ATG TGC AGG TTT GGA CCC ATG ATC CAG TGC 483  
 Leu Ile Asn Leu Ala Thr Met Cys Arg Phe Gly Pro Met Ile Gln Cys  
                   295                                  300                                  305

GAC TTG TCC TCC GAT GAC TAAGAAGTTA AAAGCAATGT TGTCATTGT 531  
 Asp Leu Ser Ser Asp Asp  
                   310

ACGTACTAAC ACATGATGTG ATAGTTTATG CTAGCTAGCT ATAACATAAG CTGTCTGTGA 591  
 GTGTGTTGTA TATTAATAAA GATCATCACT GGTGAATGGT GATCGTGTAC GTACCTACT 651  
 TAGTAGGCAA TGGAAGCACT TAGAGTGTGC TTTGTGCATG GCCTTGCCCTC TGTTTTGAGA 711  
 CTTTTGTAAT GTTTTCGAGT TTAAATCTTT GCCTTTGCCG AAAAAAAAAA AAAAAAAAAA 771  
 AAAAAA 777

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 158 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (iii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Lys Phe Thr Ile Leu Leu Ile Ser Leu Leu Phe Cys Ile Ala  
   1                                  5                                  10                                  15

His Thr Cys Ser Ala Ser Lys Trp Gln His Gln Gln Asp Ser Cys Arg  
                   20                                  25                                  30

Lys Gln Leu Gln Gly Val Asn Leu Thr Pro Cys Glu Lys His Ile Met  
                   35                                  40                                  45

Glu Lys Ile Gln Gly Arg Gly Asp Asp Asp Asp Asp Asp Asp Asp  
   50                                  55                                  60

Asn His Ile Leu Arg Thr Met Arg Gly Arg Ile Asn Tyr Ile Arg Arg  
   65                                  70                                  75                                  80

Asn Glu Gly Lys Asp Glu Asp Glu Glu Glu Glu Gly His Met Gln Lys  
                   85                                  90                                  95

Cys Cys Thr Glu Met Ser Glu Leu Arg Ser Pro Lys Cys Gln Cys Lys  
                   100                                  105                                  110

Ala Leu Gln Lys Ile Met Glu Asn Gln Ser Glu Glu Leu Glu Glu Lys  
                   115                                  120                                  125

Gln Lys Lys Lys Met Glu Lys Glu Leu Ile Asn Leu Ala Thr Met Cys  
   130                                  135                                  140

Arg Phe Gly Pro Met Ile Gln Cys Asp Leu Ser Ser Asp Asp  
   145                                  150                                  155

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 777 base pairs

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 28..501

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGCTCGTGC CGAATCGGCA CGAGAAA ATG ACC AAG TTC ACA ATC CTC CTC	51
Met Thr Lys Phe Thr Ile Leu Leu	
160 165	
ATC TCT CTT CTC TTC TGC ATC GCC CAC ACT TGC AGC GCC TCC AAA TGG	99
Ile Ser Leu Leu Phe Cys Ile Ala His Thr Cys Ser Ala Ser Lys Trp	
170 175 180	
CAG CAC CAG CAA GAT AGC TGC CGC AAG CAG CTT AAG GGG GTG AAC CTC	147
Gln His Gln Gln Asp Ser Cys Arg Lys Gln Leu Lys Gly Val Asn Leu	
185 190 195	
ACG CCC TGC GAG AAG CAC ATC ATG GAG AAG ATC CAA GGC CGC GGC GAT	195
Thr Pro Cys Glu Lys His Ile Met Glu Lys Ile Gln Gly Arg Gly Asp	
200 205 210	
GAC GAT GAT GAT GAT GAC GAC GAC AAT CAC ATT CTC AGG ACC ATG CGG	243
Asp Asp Asp Asp Asp Asp Asp Asp Asp Asn His Ile Leu Arg Thr Met Arg	
215 220 225 230	
GGA AGA ATC AAC TAC ATA CGT AAG AAG GAA GGA AAA GAC GAA GAC GAA	291
Gly Arg Ile Asn Tyr Ile Arg Lys Lys Glu Gly Lys Asp Glu Asp Glu	
235 240 245	
GAA GAA GAA GGA CAG ATG CAG AAG TGC TGC ACA GAA ATG AGC GAG CTT	339
Glu Glu Glu Gly Gln Met Gln Lys Cys Cys Thr Glu Met Ser Glu Leu	
250 255 260	
AAG AGC CCC AAA TGC CAG TGC AAA GCG CTG CAG AAG ATA ATG GAG AAC	387
Lys Ser Pro Lys Cys Gln Cys Lys Ala Leu Gln Lys Ile Met Glu Asn	
265 270 275	
CAG AGC GAG GAA CTG GAG GAG AAG GAG AAC AAG AAA ATG GAG AAG GAG	435
Gln Ser Glu Glu Leu Glu Glu Lys Glu Asn Lys Lys Met Glu Lys Glu	
280 285 290	
CTT ATG AAC TTG GCT ACT ATG TGC AGG TTT GGG CCC ATG ATC GGA TGC	483
Leu Met Asn Leu Ala Thr Met Cys Arg Phe Gly Pro Met Ile Gly Cys	
295 300 305 310	
GAC TTG TCC TCC GAT GAC TAAGAAGTTA AAAGCAATGT TGTCACTTGT	531
Asp Leu Ser Ser Asp Asp	
315	
ACGTACTAAC ACATGATGTG ATAGTTTATG CTAGCTAGCT ATAACATAAG CTGTCTCTGA	591
GTGTGTTGTA TATTAATAAA GATCATCACT GGTGAATGGT GATCGTGTAC GTACCCTACT	651
TAGTAGGCAA TGGAAGCACT TAGAGTGTGC TTTGTGCATG GCCTTGCCCTC TGTTTGTAGA	711
CTTTTGTAAAT GTTTTCGAGT TTAAATCTTT GCCTTTGCGG AAAAAAAAAA AAAAAAAAAA	771

AAAAAA

777

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 158 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Thr Lys Phe Thr Ile Leu Leu Ile Ser Leu Leu Phe Cys Ile Ala
 1           5           10           15
His Thr Cys Ser Ala Ser Lys Trp Gln His Gln Gln Asp Ser Cys Arg
      20           25           30
Lys Gln Leu Lys Gly Val Asn Leu Thr Pro Cys Glu Lys His Ile Met
      35           40           45
Glu Lys Ile Gln Gly Arg Gly Asp Asp Asp Asp Asp Asp Asp Asp
 50           55           60
Asn His Ile Leu Arg Thr Met Arg Gly Arg Ile Asn Tyr Ile Arg Lys
 65           70           75           80
Lys Glu Gly Lys Asp Glu Asp Glu Glu Glu Glu Gly Gln Met Gln Lys
      85           90           95
Cys Cys Thr Glu Met Ser Glu Leu Lys Ser Pro Lys Cys Gln Cys Lys
      100          105          110
Ala Leu Gln Lys Ile Met Glu Asn Gln Ser Glu Glu Leu Glu Glu Lys
      115          120          125
Glu Asn Lys Lys Met Glu Lys Glu Leu Met Asn Leu Ala Thr Met Cys
      130          135          140
Arg Phe Gly Pro Met Ile Gly Cys Asp Leu Ser Ser Asp Asp
      145          150          155

```

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGCCGCAA GCAGCTTAAG GGGGTGAACC TC

32

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 40 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAAGAATCA ACTACATACG TAAGAAGGAA GGAAAAGACG

40

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 44 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTGCACAGA AATGAGCGAG CTTAAGAGCC CCAAATGCCA GTGC

44

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 48 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGAGGAGAAG GAGAAGAAGA AAATGGAGAA GGAGTTCATG AACTTGGC

48

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 37 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCAGGTTTGG GCCCATGATC GGGTGCGACT TGTCCTC

37

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCACGAGTCA TGACCAAGTC ACAATTCTC

29

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCCTCCGATG ACTGAGTTAA CAAAAAAGT ACTAC

35

**WHAT IS CLAIMED IS:**

1.

5 An isolated and purified DNA molecule comprising a  
preselected DNA segment encoding a seed storage protein.

2.

The DNA molecule of Claim 1 wherein the seed storage  
protein is a soybean seed storage protein.

3.

10 The DNA molecule of Claim 1 wherein the soybean seed  
storage protein is an albumin.

4.

The DNA molecule of Claim 1 wherein the preselected DNA  
segment encodes a protein having SEQ ID NO: 2.

15

5.

The DNA molecule of Claim 1 wherein the preselected DNA  
segment has at least about (60%) identity with SEQ ID NO: 1,  
between base pairs 10 through 474.

6.

20 The DNA molecule of Claim 1 wherein the preselected DNA  
segment is SEQ ID NO: 1, modified by site-directed  
mutagenesis, such that the nutritional quality of the  
protein encoded is enhanced.

7.

25 The DNA molecule of Claim 1 wherein the preselected DNA  
segment encodes a protein having SEQ ID NO: 4.

8.

30 The DNA molecule of Claim 1 wherein the preselected DNA  
segment has at least about 60% identity with SEQ ID NO: 3,  
between base pairs 28 through 501.

9.

35 The DNA molecule of Claim 1 wherein the preselected DNA  
segment is SEQ ID NO: 3, modified by site-directed  
mutagenesis, such that the nutritional quality of the  
protein encoded is enhanced.

10.

The DNA molecule of Claim 1 wherein the preselected DNA segment encodes a protein having SEQ ID NO: 5.

11.

5 An expression cassette comprising a preselected DNA segment encoding a soybean seed storage protein, operably linked to a promoter functional in a host plant cell.

12.

10 The expression cassette of Claim 11 wherein the promoter is a seed-specific promoter.

13.

A method of increasing the level of preselected amino acid in the seed of a plant, comprising:

- 15 a) introducing into the cells of the plant an expression cassette comprising a preselected DNA segment encoding a soybean seed storage protein comprising at least one preselected amino acid, operably linked to a promoter functional in the cells of the plant to yield transformed plant cells;
- 20 b) regenerating a transformed plant from the transformed cells; and
- 25 c) isolating seeds from the regenerated transformed plant in which the seeds comprise the seed storage protein in an amount sufficient to increase the amount of the preselected amino acid in seeds of the transformed plant relative to the amount of the preselected amino acid in seeds of a corresponding untransformed plant.
- 30

14.

35 The method according to Claim 13 wherein the plant is soybean.



15.

The method according to Claim 14 wherein the preselected amino acid is lysine.

16.

5 The method according to Claim 15 wherein the preselected amino acid is methionine or cysteine, in addition to lysine.

17.

10 The method according to Claim 16 wherein the amount of lysine in the seed is increased by at least about 5-10%.

18.

The method according to Claim 17 wherein the amount of methionine and cysteine in the seed is increased by at least about 15-30%.

15

19.

A seed produced by the method of Claim 13.

20.

A plant produced from the seed of Claim 19.

21.

20 A fertile transgenic plant containing an isolated preselected DNA segment comprising a promoter and encoding a soybean seed storage protein, which comprises at least one preselected amino acid selected from the group consisting of methionine, cysteine, and lysine, under the control of the  
25 promoter, wherein the DNA segment is expressed as the seed storage protein so that the level of a seed storage protein amino acid in the seeds of the transgenic plant is increased above the level in the seeds of a soybean plant which only differ from the seeds of the transgenic plant in that the  
30 DNA segment is not artificially introduced, and wherein the DNA segment is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

22.

35 An antibody capable of specifically binding soybean albumin.

23.

The antibody of Claim 22 which is capable of specifically binding a protein having SEQ ID NO: 2 or SEQ ID NO: 4.

5

24.

A protein encoded by a preselected DNA segment contained in an isolated and purified DNA molecule; according to Claim 4.

25.

10 The protein of Claim 24 characterized in that the protein has SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 5.

26.

15 A method for isolating and purifying 2S albumins comprising the step of separating the albumins from contaminating proteins by specifically interacting the albumins with the matrix of a carbohydrate resin.

27.

The method of Claim 26 wherein the carbohydrate is a dextran.

20

AL1 N-terminal amino acid sequence by Edman degradation  
 SKWQQHQQES?REQLKGIN  
 small chain

AL2 N-terminal amino acid sequence by Edman degradation  
 SKWFQQHQQES?REQLKGINLNP7E7IM  
 small chain

P9330 (pAL1\_42, partial aa sequence deduced from cDNA)  
SKWQOHOQOESCREOLKGINLNPCEHIMEKIQAGRRGEDGSDEDHILRTMPGRINYIRKKEGKEEEEEEGHMOKCCSEMSELKSPI...

AL3 (aa sequence by Edman degradation)  
 SKWQHQQDS?RKQLQGVNLS?EKGHIME  
 small chain

P9331 (pAL1\_49, partial aa sequence deduced from cDNA)  
SKWQOHOQDSCRKOLQGVNLTCEKHIMEKIQGRGDDDDDDDDNHILRTMRGRINYIRNEGKDEDEEEEGHMOKCCCTEMSELRS...

YIRKKEGKEEEEEEGHMOK?SEM  
 large chain

YIRKKEGKEEEEEEGHMOK?SEMSELK  
 large chain

EGKDEDEEEEGHMOK?  
 large chain

1/6

FIG. 1

2/6

**FIG. 2**



4/6

1 GAG CTC GTG CCG AAT CGG CAC GAG AAA M T K F T I L  
 49 CTC ATC TCT CTC TTC TGC ATC GGC CAC ACT TGC AGC GCC ACA ATC CTC  
 97 TGG CAG CAC CAG CAA GAT AGC TGC CGC AAG CAG CTT AAG GGG GTG AAC  
 145 CTC ACG CCC TGC GAG AAG CAC ATC ATG GAG AAG ATC CAA GGC CGC GGC  
 193 GAT GAC GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT  
 241 CGG GGA AGA ATC AAC TAC ATA CGT AAG AAG AAG GAA GAA GAA GAA GAA  
 289 GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA  
 337 CTT AAG AGC CCC AAA TGC CAG TGC AAA GCG CTG CAG AAG ATA ATG GAG  
 385 AAC CAG AGC GAG GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA GAA  
 433 GAG CTT ATG AAC TTG GCT ACT ATG TGC AGG TTT GGG CCC ATG ATC GGA  
 481 TGC GAC TTG TCC TCC GAT GAC TAA GAA GTT AAA AGC AAT GTT GTC ACT  
 529 TGT ACG TAC TAA CAC ATG ATG TGA TAG TTT ATG CTA GCT AGC TAT AAC  
 577 ATA AGC TGT CTC TGA GTG TGT TGT ATA TTA ATA AAG ATC ATC ACT GGT  
 625 GAA TGG TGA TCG TGT ACG TAC CCT ACT TAG TAG GCA ATG GAA GCA CTT  
 673 AGA GTG TGC TTT GTG CAT GGC CTT GCC TCT GTT TTG AGA CTT TTG TAA  
 721 TGT TTT CGA GTT TAA ATC TTT GCC TTT GCG GAA AAA AAA AAA AAA  
 769 AAA AAA AAA

FIG. 4

5/6

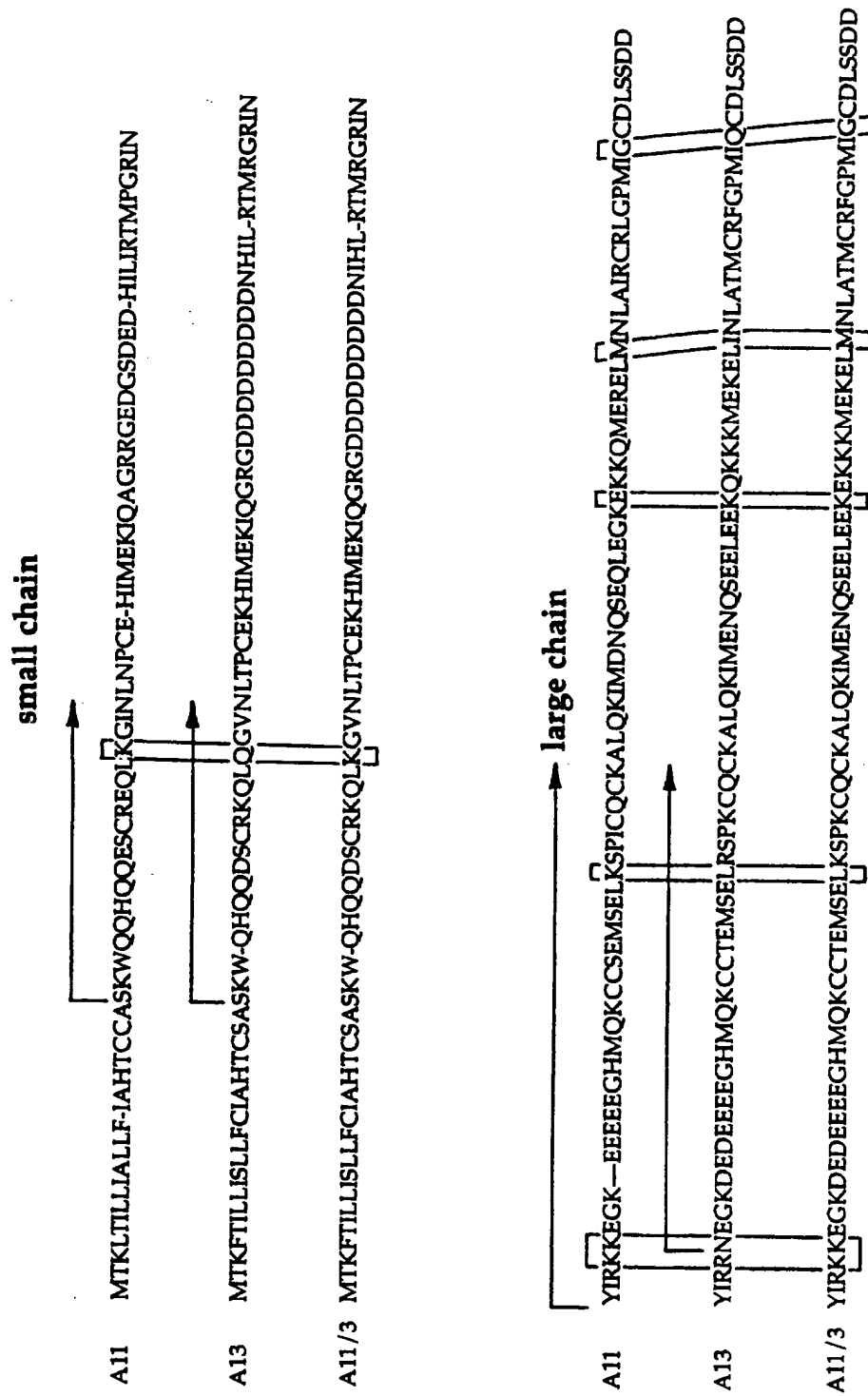
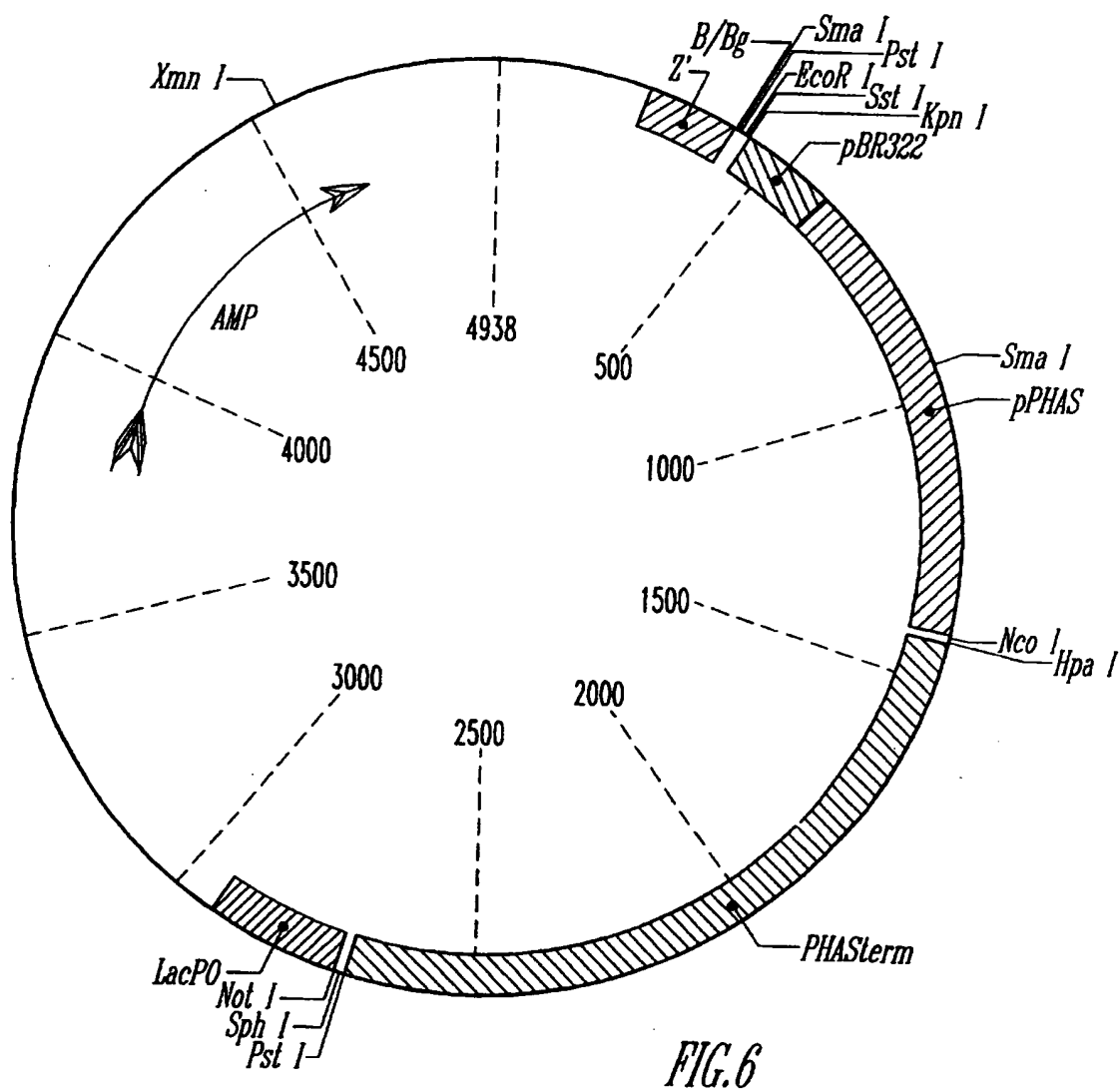


FIG. 5

6/6





**OFFICIAL**

This facsimile message and its contents are legally privileged and confidential information intended solely for the use of the addressee. If the reader of this message is not the intended recipient, you are hereby notified that any dissemination, distribution, copying or other use of this message and its contents is strictly prohibited. If you have received this telecopy in error, please notify us immediately by telephone and return the original message to us at the address shown below via the Postal Service. Thank You.

**ALSTON & BIRD LLP**

3201 Beechleaf Court, Suite 600  
Raleigh, NC 27604-1062

919-862-2200  
Fax: 919-862-2260

**RECEIVED**  
**CENTRAL FAX CENTER**

**DEC 18 2003**

**TELECOPY**

**PLEASE DELIVER AS SOON AS POSSIBLE**

**DATE:** December 18, 2003

**TO:** U.S. Patent and Trademark Office

**FROM:** Leigh W. Thorne, Reg. No. 47,992

**Re:** Appl. No. 09/478,567  
Atty Dckt No. 35718/193735

Attached please find copies of an IDS and Form 1449 that were mailed on August 12, 2003. Also, please find copies of an Amendment that was filed on August 14, 2003 in response to a Rejection dated June 17, 2003; and a copy of an IDS and 1449 that were filed on January 6, 2000.

We understand that the PTO has not received these documents. Applicant respectfully requests submission of said documents.

**NO. OF PAGES:** 22  
(Including cover page)

**OPERATOR:** A 1/4752

**IF NOT RECEIVED PROPERLY, PLEASE NOTIFY ME IMMEDIATELY AT 919-862-2233**

<b>USER CODE:</b>	THORL	<b>FAX NUMBER:</b>	703 872 9306
<b>CLIENT/MATTER:</b>	35718/193735		
<b>REQUESTED BY:</b>	Lynda	<b>VOICE NUMBER:</b>	703 305 5417

Alston & Bird

Commissioner For Patents  
Alexandria, VA 22313

Date Mailed: August 14, 2003  
Atty. Dkt. No. 035718/193735

**COPY**

Application No. 09/478,567; Filing Date: January 6, 2000  
Inventor(s): Rao *et al.*; Title of Invention: "COMPOSITIONS AND METHODS  
FOR ALTERING AMINO ACID CONTENT OF PROTEINS"  
Documents Enclosed: Amendment (13 pages); Copy of IDS filed on January 6,  
2000 (1 page); Copy of Form 1449 filed on January 6, 2000 (2 pages); and Copy  
of stamped postcard (1 page)

Check Enclosed: \$0

Kindly acknowledge receipt by placing office stamp hereon and  
returning postcard to: Lynda-Jo Pixley - ALSTON & BIRD LLP



DEC 18 2003

PATENT  
**OFFICIAL**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Appl. No.: 09/478,567  
Applicant(s): Rao *et al.*  
Filed: January 6, 2000  
Art Unit: 1638  
Examiner: R. Kallis  
Title: COMPOSITIONS AND METHODS FOR ALTERING  
AMINO ACID CONTENT FOR PROTEINS

Confirmation No.: 1859

Docket No.: 5718-16B (35718/193735)  
Customer No.: 29122

August 14, 2003

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**AMENDMENT  
37 C.F.R. § 1.121**

Sir:

In response to the Office Action dated June 17, 2003, please amend the above-identified application as follows:

**Amendments to the Specification** begin on page 2 of this paper.

**Amendments to the Claims** are reflected in the listing of claims beginning on page 5 of this paper.

**Remarks/Arguments** begin on page 8 of this paper.

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 2

Amendments to the Specification:

In the specification:

Please revise the first full paragraph beginning on page 3, line 2, as follows:

~~Fig. Figures 1A and 1B shows show~~ VSP homologies between vegetative storage protein (VSP) and other proteins, as follows.

Please revise the first full paragraph beginning on page 3, line 3, as follows:

VSP-b (same as VSP $\beta$ ) and VSP-a (same as VSP $\alpha$ ): Staswick, P.E., (1988), Plant Physiol. 87, 250-254. The amino acid sequence of the VSP-b protein is set forth in SEQ ID NO:1, and the amino acid sequence of the VSP-a protein is set forth in SEQ ID NO:2.

Please revise the first full paragraph beginning on page 3, line 6, as follows:

T.phos (tomato acid phosphatase): Erion, J.L., Ballo, B., May, L., Bussell, J., Fox, T.W., & Thomas, S.R., SwissProt database accession number P27061. The amino acid sequence of this protein is set forth in SEQ ID NO:3.

Please revise the first full paragraph beginning on page 3, line 9, as follows:

Ph.vulg (Phaseolus vulgaris): Zhon, P-Y., Tanaka, T., Yamauchi, D., & Minamikawa, T. (1997), Plant Physiol. 113, 479-485. The amino acid sequence of this protein is set forth in SEQ ID NO:4.

Please revise the first full paragraph beginning on page 3, line 12, as follows:

Ar.VSP (Arabidopsis thaliana): Yu, D.Y., Quigley, F., & Mache, R., EMBL database accession number X79490. The amino acid sequence of this protein is set forth in SEQ ID NO:5.

Please revise the first full paragraph beginning on page 3, line 15, as follows:

Ar.1A-1, Ar.17A-1 (Arabidopsis thaliana, floral organs): Utsugi, S., Sakamoto, Ogura, Y., Murata, M., & Motoyoshi, F. (1996) Plant Mol. Biol. 32, 759-765. The amino acid sequence of

RTA01/2140598v1

Appl. No.: 09/478,567

Filed: January 6, 2000

Page 3

the "Ar1A-1" protein is set forth in SEQ ID NO:6, and the amino acid sequence of the "Ar17A-1" protein is set forth in SEQ ID NO:7.

Please revise the first full paragraph beginning on page 3, line 18, as follows:

Fig. 2 shows proposed VSP $\beta$  methionine-enriched variants. The amino acid sequence of the "VSP $\beta$ -Met10" protein is set forth in SEQ ID NO:8, the amino acid sequence of the "VSP $\beta$ -Met20" protein is set forth in SEQ ID NO:9, and the amino acid sequence of the "VSP $\beta$ -Met30" protein is set forth in SEQ ID NO:10.

Please revise the first full paragraph beginning on page 3, line 23, as follows:

Fig. 4 shows the VSP $\beta$ -met10 nucleotide sequence. The VSP $\beta$ -met10 nucleotide sequence is also set forth in SEQ ID NO:11.

Please revise the Abstract, beginning on page 27, line 5, as follows:

Methods and compositions for altering amino acid composition of a protein of interest are provided, particularly proteins whose three-dimensional structure is unknown. The method comprises creating interacting molecules to the native protein and selecting for engineered proteins which retain the native conformation by antibody binding. In this manner, the levels of essential amino acids in a protein can be increased yet the biological activity of the protein maintained. Also provided is an exemplary plant protein--*Glycine max* vegetative storage protein (VSP)--in which methionine levels have been increased.

Please revise the first full paragraph beginning on page 8, line 14, as follows:

The transcriptional cassette will include the in 5'-3' 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase

RTA01/2140593v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 4

termination regions. See also, Guerincau et al., (1991) Mol. Gen. Genet. 262:141-144; Proudfoot (1991) Cell 64:671-674; Sanfacon et al. (1991) Genes Dev. 5:141-149; Mogen et al. (1990) Plant Cell 2:1261-1272; Munroe et al. (1990) Gene 91:151-158; Ballas et al. 1989) Nucleic Acids Res. 17:7891-7903; Joshi et al. (1987) Nucleic Acid Res. 15:9627-9639.

Please revise the first full paragraph beginning on page 18, line 4, to read as follows:

(a) Conserved residues (highlighted-in-blue shown in Fig. 1) were defined as those residues occurring in more than 5 of the 7 homologs. These were not targeted for substitution. The exceptions were: at residue numbers 19, 37, 146 and 179 (one of the homologs contained a methionine residue); at positions 67, 80, 130 and 169 (conserved hydrophobic amino acid exchanges observed in at least one sequence) and at position 50 (non-conservative changes from Asn to Ser/Cys in two sequences).

Please revise the first full paragraph beginning on page 21, line 6, as follows:

Fifty *E. coli* colonies containing randomly mutated VSP $\beta$  genes were picked as small patches to an SB agar plate containing glucose and ampicillin. Patches were allowed to grow overnight at ~~37°C~~ 37°C and were then transferred to a nitrocellulose filter. On the surface of an SB agar plate containing ampicillin and IPTG, this filter was placed on top (cell-side up) of a separate blocked filter to which the antigen (e.g., VSP $\alpha$ ) had been coated. During an overnight incubation at ~~30°C~~ 30°C, the cells expressed the VSP $\beta$  variant they encoded. These proteins were able to diffuse through the top filter and, if correctly folded, bind the antigen-coated filter below. The next day, the antigen-coated filter was washed with PBS-0.05% [tween] Tween<sup>TM</sup> and incubated with HRP/anti-e tag conjugate. Since the VSP $\beta$  mutants are cloned into the pCANTAB-5E vector which fuses a C-terminal epitope tag (e-tag) to the VSP $\beta$  protein variants, bound proteins were detected by this antibody in combination with enhanced chemiluminescence detection.

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 5

---

Amendments to the Claims:

1. (currently amended) A Nucleic ~~nucleic~~ acid molecule encoding comprising a nucleotide sequence which encodes an engineered protein having comprising an amino acid sequence which differs from the amino acid sequence of a native protein by at least one essential amino acid residue, wherein said engineered protein has an altered amino acid composition in comparison to said native protein, wherein said altered amino acid composition comprises an increase in essential amino acid content to at least about 5% has been altered by introducing amino acid changes into said protein; and wherein said engineered protein retains the conformation of the native protein and therefore binds to an at least one interacting molecule antibody, monoclonal antibody, antibody fragment, protein, or modified protein which is capable of binding with a to said corresponding native protein, wherein said native protein is VSP $\alpha$  or VSP $\beta$ .

2. (currently amended) The nucleotide-sequence nucleic acid molecule of claim 1, wherein said altered amino acid changes composition comprises an increase in the levels of at least one essential amino acid content to at least about 10% in the protein.

3. (currently amended) The nucleotide-sequence nucleic acid molecule encoding of claim 2 1 wherein said altered amino acid composition comprises an increase in the content of an essential amino acid is selected from the group consisting of methionine, tryptophan, lysine, valine, phenylalanine, isoleucine, leucine, threonine and cysteine.

4. (canceled)

5. (currently amended) A transformed plant containing within its genome the nucleotide sequence of Claim 2 1.

RTA01/2140598v1

Appl. No.: 09/478,567

Filed: January 6, 2000

Page 6

6. (original) A transformed plant containing within its genome the nucleotide sequence of Claim 3.

7. (currently amended) A-transformed The plant of claim 5, wherein said plant is a monocot containing within its genome the nucleotide sequence of Claim 4.

8. (currently amended) A stably transformed plant having inserted into its genome a nucleotide sequence which encodes chimeric gene-said gene-encoding an engineered protein comprising an amino acid sequence which differs from the amino acid sequence of a native protein by at least one essential amino acid residue having altered amino acid composition wherein said protein, wherein said engineered protein has an altered amino acid composition in comparison to said native protein, wherein said altered amino acid composition comprises an increase in essential amino acid content to at least about 5% and wherein said engineered protein retains the conformation of the native protein and therefore binds to an interacting molecule at least one antibody, monoclonal antibody, antibody fragment, protein, or modified protein which binds with is capable of binding to said a-corresponding native protein, wherein said native protein is VSP $\alpha$  or VSP $\beta$ .

9. (currently amended) The plant of Claim 8, wherein said altered amino acid changes composition comprises an increase in the levels of at least one essential amino acid content to at least about 10% in the protein.

10. (currently amended) The plant of Claim ~~[[9]]~~ 8, wherein said altered amino acid composition comprises an increase in the content of an essential amino acid ~~[[is]]~~ selected from the group consisting of methionine, tryptophan, lysine, valine, phenylalanine, isoleucine, leucine, threonine and cysteine.

RTA01/2140598v1



Appl. No.: 09/478,567

Filed: January 6, 2000

Page 7

11. (currently amended) The plant of Claim ~~[[10]]~~ 8, wherein said altered amino acid composition comprises an increase in methionine content to at least about 10% ~~essential-amino acid-is-increased-to-represent-5%-of-the-total-amino-acid-content-of-the-protein.~~

12. (currently amended) The plant of Claim ~~[[10]]~~ 8, wherein said altered amino acid composition comprises an increase in methionine content to at least about 20% ~~essential-amino acid-are-increased-to-represent-10%-of-the-total-amino-acid-content-of-the-protein.~~

13. (canceled)

14. (original) The plant of Claim 8, wherein said plant is a dicot.

15. (original) The plant of Claim 8, wherein said plant is a monocot.

16. (original) The plant of Claim 15, wherein said monocot is maize.

17. (currently amended) The plant of Claim ~~[[15]]~~ 14, wherein said dicot is soybean.

18. (currently amended) Seed A transformed seed of the plant of Claim 8.

19. (currently amended) Seed A transformed seed of the plant of Claim 15.

20. (currently amended) Seed A transformed seed of the plant of Claim 16.

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 8

### REMARKS/ARGUMENTS

Applicants acknowledge with appreciation the rejoining of the claims of Groups I and II. Applicants have amended the specification as suggested in the Office Action; accordingly, the objection to the specification should be withdrawn.

Claims 1-20 are pending in the application. Claims 4 and 13 have been canceled. Claims 1-3, 5, 7-12, and 17-20 have been amended. Support for the amendments can be found in the specification, particularly on pages 2, 4, 5, 7, 8, and 13 as well as in the original claims. No new matter has been added by way of amendment. Re-examination and reconsideration of the claims as amended are requested.

#### The Rejection of Claims Under 35 U.S.C. §112, First Paragraph, Should Be Withdrawn

The Office Action (6/17/03, page 2) has rejected claims 1-20 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description and enablement requirements. Applicants respectfully traverse these rejections.

Independent claims 1 and 8 (and therefore claims 2-3 and 5-7 which are dependent on or incorporate the limitations of claim 1 and claims 9-12 and 14-20 which are dependent on or incorporate the limitations of claim 8) have been amended for clarification and to include additional limitations. Support for the amendments can be found in the specification, particularly on pages 2, 4, 5, 7, 8, and 13 as well as in the original claims. Independent claims 1 and 8 now include the limitation that the native protein is VSP $\alpha$  or VSP $\beta$ .

The present specification discloses the amino acid sequences of VSP $\alpha$  and VSP $\beta$ , the sequences of several methionine-enriched VSP $\beta$  variants, and a nucleic acid molecule encoding an engineered protein (VSP $\beta$ -Met 10; see Figure 4). As noted in the Office Action (page 4):

Applicant teaches proposed methionine enriched VSP $\beta$  variants based on conserved amino acid residues within VSP homologues (pages 15-16 and Figure 2); positions of possible tolerated amino acid substitutions within VSP $\beta$  (pages 16-19); a strategy for isolating correctly folded methionine enriched variants of VSP $\beta$  by testing for binding to a VSP $\beta$  specific antibody (pages 19-22) and methionine enriched variant VSP $\beta$ -[Met]10 binding to wild type VSP $\beta$  specific antibodies (page 19).

RTA01/2140598v1

Appl. No.: 09/478,567

Filed: January 6, 2000

Page 9

Thus, the present specification provides exemplary nucleotide and amino acid sequences as well as guidance regarding evaluation of the functional limitations of the claims. Applicants believe that the teachings of the present specification, when considered by those of skill in the art, satisfy the written description and enablement requirements.

Accordingly, Applicants respectfully submit that the rejections of claims under 35 U.S.C. §112, first paragraph, should be withdrawn.

The Rejection of Claims Under 35 U.S.C. §112, Second Paragraph,  
Should Be Withdrawn

The Office Action (6/17/03, page 6) has rejected claims 1-20 under 35 U.S.C. §112, second paragraph, as being indefinite. Claim 1 was rejected for a variety of recitations and has been amended for clarification. Support for the amendments can be found throughout the specification, particularly on pages 4, 5, 7, 8, 13, and in the original claims. Claim 1 retains the phrase "capable of binding" to indicate that the antibody, monoclonal antibody, antibody fragment, protein, or modified protein (see, e.g., page 5 of the specification) binds to both the native protein and the engineered protein, but not at the same time. Applicants were concerned that if the term "binds" were used to describe both the binding of the engineered protein and the native protein, the claim might be interpreted to require simultaneous binding of both proteins. If this terminology is not acceptable, suggestions for acceptable terminology would be welcomed. Applicants also note that the term "essential amino acid" is discussed in the specification, for example, on page 7. Applicants respectfully believe that in view of the amendments to claim 1, this rejection should be withdrawn.

Claim 2 was rejected for lack of comparative basis. As this claim has been amended for clarification and is dependent on claim 1 which has also been amended for clarification, Applicants believe that this rejection should be withdrawn.

Similarly, claim 3 has been amended for clarification and claims 4 and 13 have been amended to clarify that the particular vegetative storage protein of the claim is VSP $\alpha$  or VSP $\beta$ , as discussed throughout the specification. Claim 8 has been amended for clarification. Support for these amendments can be found throughout the specification, particularly on pages 4, 5, 7, 8, 13,

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 10

and in the original claims. Claims 9, 11, 12, and 13, which are dependent on amended independent claim 8, have also been amended for clarification. Support for these amendments can be found throughout the specification, particularly on page 7, and in the original claims. Claim 17 has been amended to correct dependency, and claims 18-20 have been amended to add an article. In view of the above amendments and comments, Applicants respectfully submit that the rejections of claims under 35 U.S.C. §112, second paragraph, should be withdrawn.

The Rejection of Claims Under 35 U.S.C. §101 Should Be Withdrawn

The Office Action (6/17/03, page 8) rejected claims 18-20 under 35 U.S.C. §101 as lacking utility because "[t]he seeds of Claims 18-20 encompass untransformed seeds." These claims have been amended to require that the seeds are transformed. Accordingly, this rejection has been obviated by amendment and should be withdrawn.

The Rejection of Claims Under 35 U.S.C. §102(b) Should Be Withdrawn

The Office Action (6/17/03, page 9) has rejected claims 1-14, 17, and 18 under 35 U.S.C. §102(b) as anticipated by Jung *et al.* WO 97/35023, published September 25, 1997.

Applicants respectfully traverse this rejection. Applicants believe that the reference is not citable against the present application under 35 U.S.C. §102(b). The Jung reference has an international publication date of September 25, 1997, whereas the present application has a priority date of December 10, 1997, less than one year later. Accordingly, the Jung reference cannot be cited against the present application under 35 U.S.C. §102(b).

Independent claims 1 and 8 (and therefore claims 2-3 and 5-7 which are dependent on or incorporate the limitations of claim 1 and claims 9-12 and 14-20 which are dependent on or incorporate the limitations of claim 8) have been amended to include additional limitations. Particularly, independent claims 1 and 8 now include the limitation that the native protein is VSP $\alpha$  or VSP $\beta$ . The Jung reference does not teach or suggest this limitation and therefore cannot anticipate the subject matter of the claims.

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 11

---

Further, Applicants note that the assignee on the Jung reference is the same assignee as the one on the present application and therefore the Jung reference is not available as a reference against the present claims under 35 U.S.C. §103(c).

In view of the above amendments and arguments, Applicants respectfully submit that the rejection of the claims under 35 U.S.C. §102(b) should be withdrawn.

The Office Action (6/17/03, page 10) has rejected claims 1-14, 17, and 18 under 35 U.S.C. §102(e) as anticipated by Tarczyński *et al.* U.S. Pat. No. 6,080,913, filed September 25, 1996.

Independent claims 1 and 8 (and therefore claims 2-3 and 5-7 which are dependent on or incorporate the limitations of claim 1 and claims 9-12 and 14-20 which are dependent on or incorporate the limitations of claim 8) have been amended to include additional limitations. Particularly, independent claims 1 and 8 now include the limitation that the engineered protein retains the conformation of the native protein, which is VSP $\alpha$  or VSP $\beta$ . The Tarczyński reference does not teach or suggest this limitation and therefore cannot anticipate the subject matter of the claims.

Further, Applicants note that the assignee on the Tarczyński reference is the same assignee as the one on the present application and therefore the Tarczyński reference is not available as a reference against the present claims under 35 U.S.C. §103(c).

In view of the above amendments and arguments, Applicants respectfully submit that the rejection of the claims under 35 U.S.C. §102(e) should be withdrawn.

#### The Rejection of Claims Under 35 U.S.C. §103 Should Be Withdrawn

The Office Action (6/17/03, page 11) has rejected Claims 1-20 under 35 U.S.C. §103(a) over the Jung reference in view of Gordon-Kamm *et al.* (1990) *The Plant Cell* 2: 603-608. The Office Action states that "[t]he claims are drawn to soybean and maize plants transformed with a nucleic acid molecule encoding a vegetative storage protein...."

RTA01/2140598v1

Appl. No.: 09/478,567

Filed: January 6, 2000

Page 12

---

Applicants respectfully traverse this rejection. Applicants note that claims 1-4 are not drawn to transformed plants and therefore should apparently not have been included in this rejection. In addition, the claims that are drawn to transformed plants are not limited to maize and soybean, as stated in the Office Action. Applicants also note that the Jung reference has the same assignee as the present application and therefore the Jung reference is not available to be cited against the present application under 35 U.S.C. §103(c).

Accordingly, Applicants respectfully submit that the rejection of claims under 35 U.S.C. §103(c) should be withdrawn.

#### Consideration Of Previously Submitted Information Disclosure Statement

It is noted that an initialed copy of the PTO Form 1449 that was submitted with Applicants' Information Disclosure Statement filed January 6, 2000, has not been returned to Applicants' representative with the Office Action. Accordingly, it is requested that an initialed copy of the Form 1449 be forwarded to the undersigned with the next communication from the PTO. In order to facilitate review of the references by the Examiner, a copy of the Information Disclosure Statement and the Form 1449 are attached hereto. Copies of the cited references were provided at the time of filing the original Information Disclosure Statement, and, therefore, no additional copies of the references are submitted herewith. Applicants will be pleased to provide additional copies of the references upon the Examiner's request if it proves difficult to locate the original references.

#### CONCLUSION

In view of the above amendments and remarks, Applicants submit that the rejections of the claims under 35 U.S.C. §§112, 101, 102, and 103 are overcome. Applicants respectfully submit that this application is now in condition for allowance. Early notice to this effect is solicited.

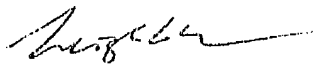
If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned.

RTA01/2140598v1

Appl. No.: 09/478,567  
Filed: January 6, 2000  
Page 13

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

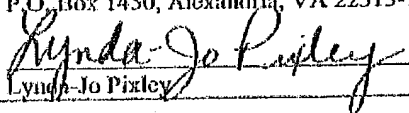


Leigh W. Thorne  
Registration No. 47,992

Customer No. 29122  
**ALSTON & BIRD LLP**  
Bank of America Plaza  
101 South Tryon Street, Suite 4000  
Charlotte, NC 28280-4000  
Tel Raleigh Office (919) 862-2200  
Fax Raleigh Office (919) 862-2260

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Non-Fee Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on August 14, 2003

  
Lynda Jo Pixley

RTA 2140598v1

RTA01/2140598v1

Attorney's Docket No. 5716-16B

PATENT

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Rao et al.  
Appl No.: To be assigned  
Filed: Concurrently Herewith  
For: COMPOSITIONS AND METHODS FOR ALTERING  
AMINO ACID CONTENT OF PROTEINS  
Group Art Unit: To be assigned  
Examiner: To be assigned  
January 6, 2000

COPY

Assistant Commissioner for Patents  
Washington, DC 20231

INFORMATION DISCLOSURE STATEMENT  
CITATION UNDER 37 C.F.R. § 1.97

Sir:

Attached is a list of documents on form PTO-1449. It is requested that the Examiner consider these documents and officially make them of record in accordance with the provisions of 37 C.F.R. § 1.97 and Section 609 of the MPEP. By submitting the listed documents, Applicant in no way makes any admission as to the prior art status of the listed documents, but is instead submitting the listed documents for the sake of full disclosure.

All items are attached except those that were supplied in parent Application No. 08/988,015 filed 12/10/1997. Since the benefit of this application was claimed under 35 U.S.C. 120, no copies need to be furnished in accordance with 37 C.F.R. 1.98(d); however, copies will be furnished on request.

Respectfully submitted,

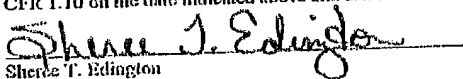


W. Murray Spruill  
Registration No. 32,943

ALSTON & BIRD LLP  
Post Office Drawer 34009  
Charlotte, NC 28234  
Tel Raleigh Office (919) 420-2200  
Fax Raleigh Office (919) 420-2260

"Express Mail" Mailing Label Number EL039496705US  
Date of Deposit: January 6, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner of Patents, Washington, DC 20231.

  
Sherice T. Edington



Substitute for form 1449A/PTO  <b>INFORMATION DISCLOSURE STATEMENT BY APPLICANT</b>  (Use as many sheets as necessary)				<b>Complete if known</b>	
				Application Number	To be assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Rao et al.
				Group Art Unit	To be assigned
				Examiner Name	To be assigned
Sheet	1	of	2	Attorney Docket Number	5718-16B

COPY

U. S. PATENT DOCUMENTS						
Examiner Initials*	Cite No.	U.S. Patent Document		Name of Patentee or Applicant Of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Pages, Columns, Lines, Where Relevant Passages of Relevant Figures Appear
		Number	Kind Code (if known)			
	1	5,589,615		De Clercq et al.	12/31/1996	
	2	5,559,223		Falco et al.	09/24/1996	

FOREIGN PATENT DOCUMENTS									
Examiner Initials	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T	
		Office	Number	Kind Code (if known)					
	3	WO	93/08682		State University of New Jersey - Rutgers	05/13/1993			
	4	WO	94/16088		Rijksuni-Versiteit Leiden	07/21/1994			
	5	WO	95/22625		Affymax Technologies	08/24/1995			
	6	WO	95/31554		E.I. DuPont De Nemours and Company	11/23/1995			
	7	WO	96/38563		Pioneer Hi-Bred International, Inc.	12/05/1996			
	8	WO	97/35023		Pioneer Hi-Bred International, Inc.	09/25/1997			
	9	WO	97/20078		Affymax Technologies	06/05/1997			

Examiner Signature		Date Considered	
-----------------------	--	--------------------	--

\*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute for form 1449A/PTO  <b>INFORMATION DISCLOSURE STATEMENT BY APPLICANT</b>  (Use as many sheets as necessary)				<b>Complete . . . Known</b>	
				Application Number	To be assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Rao et al.
				Group Art Unit	To be assigned
Examiner Name	To be assigned	COPY			
Attorney Docket Number	5718-16B				
Sheet	2	of	2		

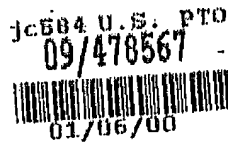
NON PATENT LITERATURE DOCUMENTS				
Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issn number(s), publisher, city and/or country where published.	T	
	10	ALTENDACH et al., Enhancement of the Methionine Content of Seed Proteins by the Expression of a Chimeric Gene Encoding a Methionine-Rich Protein in Transgenic Plants, Plant Molecular Biology, 1989, pages 513-522, vol. 13, Belgium		
	11	SCOTT et al., Searching for Peptide Ligands with an Epitope Library, Science, 1990, pp. 386-390, vol. 249		
	12	STASWICK, P., Novel Regulation of Vegetative Storage Protein Genes, The Plant Cell, January 1990, pages 1-6, vol. 2,		
	13	STEMMER, W., Rapid Evolution of a Protein <i>In Vitro</i> By DNA Shuffling, Nature, Aug. 4, 1994, pages 389-391, vol. 374		
	14	STEMMER, W., DNA Shuffling by Random Fragmentation and Reassembly: <i>In Vitro</i> Recombination for Molecular Evolution, Proc. Natl. Acad. Sci. USA, Oct. 1994, ppages 10747-10751, vol. 91		
	15	HONGDI et al., A Phage Display System for Studying the Sequence Determinations of Protein Folding, Protein Science, 1995, pp. 1108-1117, vol. 4		
	16	MARCELLINO et al., Modified 2S Albumins with Improved Tryptophan Content are Correctly Expressed in Transgenic Tobacco Plants, FEBS Letters, 1996, pages 154-158, vol. 385		
	17	MOLVIG et al., Enhanced Methionine Levels and Increased nutritive Value of Seeds of Transgenic Lupinus ( <i>Lupinus Angustifolius</i> L.) Expressing a Sunflower Seed Albumin Gene, Proc. Natl. Acad. Sci. USA, Aug. 1997, pages 8393-8398, Vol. 94, Agricultural Sciences		
	18	FRIGUET et al., Immunochemical Analysis of Protein Conformation, Protein Structure a Practical Approach, pp. 287-310, IRL Press, Oxford University Press, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK		

RYA01/2072075v1

Examiner Signature		Date Considered	
-----------------------	--	--------------------	--

\*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

copy



Assistant Commissioner For Patents  
Washington, DC 20231

Date Mailed: 01/06/2000  
Atty. Dkt. No. 5718-16B

Kindly acknowledge receipt of the accompanying DIVISIONAL PATENT  
APPLICATION with Application Transmittal Cover Sheet for:

Inventor(s): Rao et al.

Title of Invention: COMPOSITIONS AND METHODS FOR ALTERING  
AMINO ACID CONTENT OF PROTEINS

Pages of Spec. (including claims and abstract) 26; No. of Claims 20

No. of Drawing Sheets 8; Declaration Enclosed Yes - copy (4 pages)

Small Entity Statement Enclosed N/A;

IDS with PTO 1449 Enclosed yes-2 pages; (No. of 1449 Cites Enclosed 1)

Assignment with Cover Sheet and \$40.00 Fee Enclosed

Check(s) Enclosed \$690.00

Please place office stamp hereon and Return Postcard to : WMS/sie  
ALSTON & BIRD LLP

Commissioner For Patents  
Alexandria, VA 22313

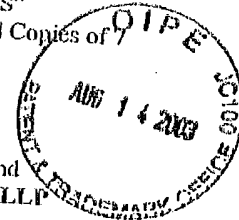
Date Mailed: Aug 12, 2003  
Atty. Dkt. No. 035718/193735

**COPY**

Application No. 09/478,567; Filing Date: January 6, 2000  
Inventor(s): Rao *et al.*; Title of Invention: "COMPOSITIONS AND METHODS  
FOR ALTERING AMINO ACID CONTENT OF PROTEINS"  
Documents Enclosed: IDS (1 page); Form 1449 (1 page); and Copies of 7  
References

Check Enclosed: \$0

Kindly acknowledge receipt by placing office stamp hereon and  
returning postcard to: Lynda-Jo Pixley - ALSTON & BIRD LLP



Attorney's Docket No. 035718/193735

PATENT

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Rao *et al.*  
Appl. No.: 09/478,567  
Filed: January 6, 2000  
For: COMPOSITIONS AND METHODS FOR ALTERING AMINO ACID  
CONTENT OF PROTEINS

Confirmation No.: 1859  
Art Unit: 1638  
Examiner: Russell Kallis

COPY

August 12, 2003

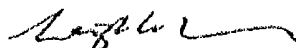
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT  
CITATION UNDER 37 C.F.R. § 1.97

Sir:

It is requested that the Examiner consider these documents and officially make them of record in accordance with the provisions of 37 C.F.R. § 1.97 and Section 609 of the MPEP. By submitting the listed documents, Applicant in no way makes any admission as to the prior art status of the listed documents, but is instead submitting the listed documents for the sake of full disclosure.

Respectfully submitted,

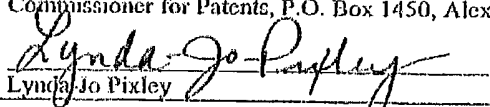


Leigh W. Thorne  
Registration No. 47,992

Customer No. 29122  
ALSTON & BIRD LLP  
Bank of America Plaza  
101 South Tryon Street, Suite 4000  
Charlotte, NC 28280-4000  
Tel Raleigh Office (919) 862-2200  
Fax Raleigh Office (919) 862-2260

## CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to:  
Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on August 12, 2003

  
Lynda Jo Pixley

RTA01/21409R7v1

Substitute for form 1449/PTO (Revised 04/2003)  <b>INFORMATION DISCLOSURE STATEMENT BY APPLICANT</b> (Use as many sheets as necessary)				<b>Complete if Known</b>		
				Application Number		
				Filing Date		
				First Named Inventor		
				Group Art Unit		
Sheet 1 of 1				Examiner Name Russell Kallis Attorney Docket Number 035718/193735		
<b>U. S. PATENT DOCUMENTS</b>						
Examiner Initials*	Cite No.	Document Number Number - Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages of Relevant Figures Appear	
	1	US-5,288,629	02-22-1994	Berkner		
	2	US-5,559,223	09-24-1996	Falco <i>et al.</i>		
<b>FOREIGN PATENT DOCUMENTS</b>						
Examiner Initials	Cite No.	Foreign Patent Document Country Code - Number Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	English Language Translation Attached
<b>OTHER DOCUMENTS</b>						
Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.				English Language Translation Attached
	3	ARNOLD, F.H., <i>et al.</i> , "Optimizing Industrial Enzymes by Directed Evolution," <i>Adv. Biochem. Eng. Biotechnol.</i> , 1997, pp. 1-14, Vol. 58.				
	4	DYER, J.M., <i>et al.</i> , "Extensive Modifications for Methionine Enhancement in the beta-Barrels Do Not Alter the Structural Stability of the Bean Seed Storage Protein Phaseolin," <i>J. Protein Chemistry</i> , 1995, pp. 665-678, Vol. 14(8).				
	5	GOLDBERG, M.E., "Investigating Protein Conformation, Dynamics and Folding with Monoclonal Antibodies," <i>TIBS</i> , 1991, pp. 358-362, Vol. 16(10).				
	6	JAYNES, J.M., "De Novo Designed Synthetic Plant Storage Proteins: Enhancing Protein Quality of Plants for Improved Human and Animal Nutrition," <i>Proceedings of Alltech's 10<sup>th</sup> Annual Symposium</i> , Lexington, KY, May 1994, pp. 129-153.				
	7	LOPES, M.A., "Approaches for Enhancing the Lysine Content of Maize Seed," <i>Biotechnol. Nutr. Proc. Int. Symp. 3<sup>rd</sup></i> , " 1992, pp. 237-252.				
Examiner Signature		Date Considered				

\*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

RTA01/2140900v1